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The Role of the Dileucine Motif in Helix VIII of the BLT1 Receptor and RhoA in Neutrophil Degranulation.

By

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Abbreviations:

LTB_4: leukotriene B_4.

PLB-BLT1 (wt): PLB-985 cells stably transfected with BLT1 cDNA.

PLB-BLT1 (2LA): PLB-985 cells stably transfected with mutant 2LA(304-305) cDNA.

GPCR: G protein-coupled receptor.

PAFR: platelet-activating factor receptor.

G418: geneticin.

EtOH: ethanol.

PTX: *Bordetella pertussis* toxin.

PP1: 4-amino-5-(4methylphenyl)-7-(*tert*-butyl)pyrazolo[3,4-*d*]pyrimidine.

AG490: *N*-benzyl-3,4-dihydroxylbenzylidenecyanoacetamide.

SB203580: 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole.

PD98059: 2'-amino-3'-methoxyflavone.

AG1478: 4-(3-chloroanilino)-6,7-dimethoxyquinazolin.

fMLP: formyl-met-leu-phe.

SUMMARY

Neutrophil degranulation involves a number of well-orchestrated structural and biochemical events. We have investigated the mechanism of intracellular signalling involved in neutrophil degranulation that was mediated by the high affinity leukotriene (LT)B_4 receptor, BLT1. The model systems used were consisted of Peripheral blood neutrophils as well as promyeloid PLB-985 cells, stably transfected with human BLT1 cDNA (PLB-BLT) or a substitution mutant (2L(304-305)/A) of the distal dileucine motif in helix VIII of BLT1, and differentiated into a neutrophil-like phenotype. The degranulation of these cells was measured in the presence and absence of factors that would affect the signaling pathway. The results show that Degranulation responses to LTB_4 were similar for differentiated PLB-BLT1 and neutrophils. However, the degranulation response of cells bearing the dileucine mutation in helix VIII of BLT1 was significantly reduced in response to LTB_4. Pretreatment of differentiated PLB-BLT1 cells and neutrophils with Y-27632, a pharmacological inhibitor of p160-ROCK, the down-stream effector of the small GTPase RhoA, abrogated their degranulation in response to LTB_4. The degranulation defect observed with the

dileucine mutation was corrected by transient transfection of the cells bearing the mutation with a constitutively active form of RhoA. Taken together, our results suggest an essential role for the distal dileucine motif in helix VIII of BLT1 involving RhoA which allows normal neutrophil degranulation in response to LTB_4.

Key words: Neutrophil, BLT1, helix VIII, distal dileucine motif, RhoA signaling, degranulation.

Introduction

1.0 Human Neutrophils

Neutrophils are key cellular elements of the innate immune system, which provide protection from invading bacteria. When normal regulatory mechanisms fail, the neutrophil is also responsible for immunologically induced tissue injury (Faist and Kim 1998). Following activation by bacterial by-products or other immune stimuli such as lipopolysaccharides, glycolipids, and methylated DNA, neutrophils execute several specialized functions that include chemotaxis, phagocytosis and the generation of reactive oxygen metabolites. All of these processes are required for the elimination of invading micro-organisms or cellular debris.

Neutrophils are highly mobile, short-lived white blood cells that are densely packed with secretory granules. They emigrate from the bone marrow into the blood and infiltrate tissues in response to injury or infection. In healthy individuals, peripheral blood neutrophils make up the majority of white blood cells (40-80%).

The lungs have the largest marginated pool of neutrophils in the body; they fulfill an important role in maintaining alveolar sterility. As a major effector cell in innate immunity neutrophils act as a "double-edged sword". If neutrophils are absent, for example in congenital neutropenia, or the more common "cyclic neutropenia" opportunistic infections result from overgrowth of normally resident skin and gut bacteria

and fungi at sites of injury, or exposed mucosal tissues. At the other extreme, accumulation and over activation of neutrophils can be fatal in disorders such as in septic shock or acute respiratory distress. The tissue-damaging effects of neutrophils are completely dependent on their degranulation.

2.0 Leukotriene B_4 (LTB_4)

2.1 Arachidonic acid

Arachidonic acid plays a central role in a biological control system where such oxygenated derivatives as prostaglandins, thromboxanes, and leukotrienes are mediators. The leukotrienes are formed by transformation of arachidonic acid into an unstable epoxide intermediate, leukotriene A_4, which can be converted enzymatically by hydration to leukotriene B4, and by addition of glutathione to leukotriene C_4. Leukotriene C4 is metabolized to leukotrienes D_4 and E_4 by successive elimination of a gamma-glutamyl residue and glycine. The Slow-reacting substance found during anaphylaxis consists of leukotrienes C_4, D_4, and E_4. The cysteinyl-containing leukotrienes are potent bronchoconstrictors which increase vascular permeability in postcapillary venules, and stimulate mucus secretion. Leukotriene B4 causes adhesion and chemotactic movement of leukocytes and stimulates aggregation, enzyme release, and generation of superoxide in neutrophils. Leukotrienes C_4, D_4, and E_4, which are released from the lung tissue of asthmatic subjects exposed to specific allergens, seem to play a pathophysiological role in immediate hypersensitivity reactions. These leukotrienes, as well as leukotriene B4, have pro-inflammatory effects.

2.2 Leukotriene B_4 (LTB_4)

LTB_4 was initially discovered by Borgeat and Samuelsson,(Borgeat and Samuelsson 1979) and found to be a potent neutrophil chemoattractant by Ford-Hutchinson et al. (Ford-Hutchinson, Bray et al. 1980). A high-affinity binding site for LTB_4 on human neutrophils was initially detected by Goldman and Goetzl in 1982 with a K_d of 0.39nM (Goldman and Goetzl 1984),others detected this binding site with K_d of 0.46nM (Lin, Ruppel et al. 1984), and 1.5nM (Bomalaski and Mong 1987). Leukotriene B_4 (LTB_4) is an extremely potent lipid inflammatory mediator derived from membrane phospholipids by the sequential actions of cytosolic phospholipase A_2, 5-lipoxygenase (5-LO) and LTA_4 hydrolase.

The major activities of LTB_4 include the recruitment and activation of leukocytes, suggesting that it has considerable functional overlap with the chemokine family of chemoattractant peptides, which also direct the recruitment of leukocytes. Though structurally completely different, the lipid LTB_4 and the peptide chemokines mediate their function through the same class of receptors, the G protein-coupled seven transmembrane domain receptor (GPCR) superfamily.

Two GPCRs for LTB_4 have been identified, BLT1 and BLT2. By mediating the activities of LTB_4, these receptors participate both in the recruitment and activation of leukocytes as part of host immune responses to invading pathogens, as well as in the pathogenesis of inflammatory diseases in which LTB_4 has been implicated.

3.0 Leukotriene B_4 receptors BLT1 and BLT2

3.1 BLT1

Molecular identification of a receptor for LTB_4 eluded investigators for many years, however, human high-affinity LTB_4 receptor was finally cloned by Yokomizo et al. in 1997 from retinoic acid-differentiated HL-60 cells using a subtraction strategy (Yokomizo, Izumi et al. 1997), and the mouse ortholog by Huang et al. (Huang, Garcia-Zepeda et al. 1998). This receptor was initially named BLTR, and subsequently renamed BLT1 when a second LTB_4 receptor was identified. Membrane fractions of COS-7 cells transfected with the BLT1 cDNA demonstrated LTB_4 binding with a K_d comparable to that observed in retinoic acid-differentiated HL-60 cells, and CHO cells stably transfected with the BLT1 cDNA demonstrated LTB_4-induced increases in intracellular calcium and chemotactic responses, indicating that this cDNA encoded the high-affinity LTB_4 receptor (Yokomizo, Izumi et al. 1997).

Interestingly, this sequence had been previously cloned using degenerate PCR strategies by two independent groups as an orphan receptor gene that appeared to encode a member of the G protein-coupled seven transmembrane domain receptor (GPCR) superfamily. At that time, the receptor was called R2 by Raport et al. (Raport, Schweickart et al. 1996) and chemoattractant receptor-like 1 (CMKRL1) by Owman et al. (Owman, Nilsson et al. 1996).

The mouse ortholog of BLT1 was independently cloned by Huang et al. In 1998, by performing degenerate PCR with primers directed to well-conserved transmembrane domains of chemoattractant GPCRs, using cDNA isolated from murine eosinophils (Huang, Garcia-Zepeda et al. 1998).

Martin et al. identified the identical murine sequence by screening a mouse genomic library with a fragment of the human cDNA identified previously as encoding the $P2Y_7$ receptor (Martin, Ronde et al. 1999).

Human BLT1 gene was localized to chromosome 14 (Raport, Schweickart et al. 1996; Owman, Nilsson et al. 1996; Akbar, Dasari et al. 1996). Kyte-Doolittle hydrophobicity analysis of the amino acid sequences of human (Owman, Nilsson et al. 1996; Raport, Schweickart et al. 1996) and mouse (Huang, Garcia-Zepeda et al. 1998) BLT1 showed the presence of seven hydrophobic transmembrane domains common to GPCRs. The amino acid sequences also contain other motifs characteristic of this family of receptors (Huang, Garcia-Zepeda et al. 1998; Owman, Nilsson et al. 1996), including (a) conserved proline residues in several of the transmembrane domains, which are thought to induce flexibility in the helix formations; (b) conserved cysteine residues in two of the extracellular loops for intra-molecular chain disulfide bonding; (c) a serine and threonine-rich C-terminal intra-cytoplasmic segment, which in other GPCRs are sites of phosphorylation involved with receptor desensitization and internalization; and (d) consensus sequences for N-

linked glycosylation near the N-terminus and in one of the extracellular loops (Murphy 1994).

Mouse BLT1 has been demonstrated to be N-linked glycosylated, as in-vitro translation of the cDNA in the presence of dog pancreatic microsomes revealed an upward shift in mobility of the protein product on SDS-PAGE of ~4kD compared with the protein product translated in the absence of microsomes (Huang, Garcia-Zepeda et al. 1998). The highly conserved DRYLAIV motif at the end of the third transmembrane region of the GPCR family of receptors is present as DRSLAV in both human and mouse BLT1 (Yokomizo, Izumi et al. 1997; Huang, Garcia-Zepeda et al. 1998) .

Human BLT1 is 352 amino acid residues in length, and mouse BLT1 is 351 amino acids, similar to the lengths of other chemoattractant receptors (Murphy 1994). The primary structures of human and mouse BLT1 are 78% identical at the amino acid level. Interestingly, the three intra-cytoplasmic loops are identical across these species. In contrast, these sequences are not conserved across the subfamily of chemoattractant receptors, suggesting that human and mouse BLT1 may be coupled to a unique, well-conserved, signaling pathway among the chemoattractant receptors (Huang, Garcia-Zepeda et al. 1998).

Leukocyte BLT1 expression is up-regulated in inflammation. Mouse BLT1 transcription, which is not detectable in resting peritoneal cells, is dramatically increased in both activated macrophages and neutrophils elicited into the peritoneum of mice by sodium

casein injection (Huang, Garcia-Zepeda et al. 1998). IFNγ and glucocorticoids have also been shown to induce BLT1 expression. Mouse BLT1 transcription is induced in the RAW 264.7 macrophage cell line by IFNγ (Huang, Garcia-Zepeda et al. 1998), and human BLT1 transcription is induced in peripheral blood neutrophils by dexamethasone (Stankova, Turcotte et al. 2002).

Table 1: Regulators of BLT1 expression

Regulators of BLT1 expression

• **Increased by**	• **Decreased by**
LTB4	**IFN-γ,**
IL-10,	**LPS,**
Dexamethasone	**TNFα**
IL-2	
IL-15	

Petterson A., et al, 2005, Stankova J. et al, 2002

3.2 BLT2

Binding studies of LTB_4 suggested that in addition to the presence of a high-affinity receptor, a second, low-affinity LTB_4 receptor was also present on human neutrophils (Goldman and Goetzl 1984; Lin, Ruppel et al. 1984) with a K_d of 61nM (Goldman and Goetzl 1984). BLT2, a second seven transmembrane spanning G protein-coupled LTB_4 receptor, was identified by Yokomizo et al. (Yokomizo, Kato et al. 2000).

Membrane fractions of HEK 293 cells transfected with the cloned human BLT2 demonstrated specific and saturable LTB_4 binding with a K_d of 22.7nM, approximately 20-fold higher than human BLT1 transfectants. CHO cells stably transfected with human BLT2 also demonstrated LTB_4-induced increases in intracellular calcium and chemotactic responses, indicating that this sequence encoded an LTB_4 receptor. This was confirmed when human BLT2 was independently cloned to be an LTB_4 receptor by three additional groups (Tryselius, Nilsson et al. 2000; Kamohara, Takasaki et al. 2000; Wang, Gustafson et al. 2000).

The two LTB_4 receptor genes form a cluster in both the human (Nilsson, Tryselius et al. 2000; Yokomizo, Kato et al. 2000) and mouse (Yokomizo, Kato et al. 2000) genomes. The human BLT2 gene being located approximately 3 kb 5′ of the human BLT1 gene, and overlaps the 5′ untranslated region of one of the 4′ identified splice variants of BLT1 (Nilsson, Tryselius et al. 2000). Both genes are transcribed in the same direction. The mouse BLT2 gene similarly is located approximately 4 kb 5′ of the BLT1 coding

sequence (Yokomizo, Kato et al. 2000). The identity between human BLT1 and BLT 2 at the amino acid level, of 45.2%, is lower than that between CXCR1 and CXCR2 (77%) or between FPR1 and FPRL1 (69%) or FPRL2 (58%), suggesting that the duplication event that generated the two LTB_4 receptors may have occurred earlier than the events that generated these other receptor clusters (Yokomizo, Kato et al. 2000).

3.3 BLT1 and BLT2 signaling

BLT1 and BLT2 can be distinguished pharmacologically. Multiple LTB_4 receptor antagonists have been developed, including CP-105,696 (Showell, Pettipher et al. 1995), CP-195,543 (Showell, Conklyn et al. 1998), U-75302 (Lawson, Wishka et al. 1989), LY255283 (Herron, Goodson et al. 1992), ZK 158252 and ONO-4057 (Kishikawa, Tateishi et al. 1992; Yokomizo, Kato et al. 2000). Some of these agents selectively antagonize BLT1 or BLT2, whereas others antagonize both receptors. CP-105,696 and U-75302 compete with LTB_4 binding in a dose-dependent manner to membrane fractions of CHO cells expressing human BLT1 but not human BLT2, LY 255283 competes with LTB_4 binding to human BLT2 but not human BLT1, and ZK 158252, CP-195,543, and ONO 4057 compete with LTB_4 binding to both receptors (Yokomizo, Kato et al. 2000; Yokomizo, Kato et al. 2001).

There are two principal signal transduction pathways involving the G-protein coupled receptors: the cAMP signal pathway and the Phosphatidylinositol signal pathway. When a ligand binds to the GPCR it causes a conformational change in the GPCR which allows it to act as a guanine nucleotide exchange factor (GEF). The GPCR can then activate an associated G-protein by exchanging its bound GDP for a GTP. The G-protein's α subunit, together with the bound GTP, can then dissociate from the β and γ subunits to further

affect intracellular signaling proteins or target functional proteins directly depending on the α subunit type ($G_{\alpha s}$,$G_{\alpha i}$, $G_{\alpha q/11}$, $G_{\alpha 12/13}$).

Table2: Characteristics of BLT1 and BLT2.

	BLT1	**BLT2**
Gene location	chromosome1 4q 11.2-q12	
Amino acids	352 amino acids	358 amino acids
Identity between human and mouse	78.6%	92.7%
Agonists	LTB4 12 epi-LTB4	LTB4 12 epi-LTB4 12(s)-HTETE 12(s)HPETE 12(R)HTETE 20-hydroxy LTB4
Pharmacologic inhibitors	CP105696 CP195543 ZK158252 U75302 ONO4057	CP195543 ZK158252 ONO4057 LY2442843

Homology between BLT1and BLT2 is 45.2%

Some differences in G protein-coupling have been observed between the two receptors. BLT1 and BLT2 receptors appear to use different G proteins in mediating different cellular events, and receptor G protein coupling may differ in different cell types. LTB_4-induced chemotaxis of CHO cells stably transfected with either human BLT1 or human BLT2 is completely abolished by *Bordetella pertussis* toxin (PTX), suggesting that chemotatic responses directed by both receptors involve $G\alpha_{i/0}$ subunit signaling. In contrast, increases in intracellular calcium induced by LTB_4 in CHO-human BLT1 and CHO-human BLT2 transfectants are only partially blocked by PTX, indicating that calcium responses directed by the two receptors are mediated by both PTX-sensitive and PTX-insensitive G proteins in CHO cells (Yokomizo, Izumi et al. 1997; Yokomizo, Kato et al. 2000).

Experiments co-transfecting BLT1 with different Gα subunits into COS-7 cells indicate that BLT1 can couple to $G\alpha_{16}$, a PTX-insensitive α subunit of the G_q class of G proteins (Gaudreau, Le Gouill et al. 1998). As opposed to the results with CHO transfectants, LTB_4-induced increase in intracellular calcium in human neutrophils was largely PTX-sensitive (Powell, MacLeod et al. 1996), suggesting that the LTB_4 receptors couple to different types of G proteins in different cell types. Both LTB_4 receptors also mediate inhibition of forskolin-induced adenylyl cyclase activity, but do so using different G proteins. Whereas inhibition of forskolin-induced adenylyl cyclase activity by LTB_4 was reduced approximately 80% by PTX in CHO-human BLT1 transfectants, PTX had a

negligible effect on LTB_4-induced inhibition of forskolin-induced adenylyl cyclase activity in CHO-human BLT2 transfectants (Yokomizo, Kato et al. 2000).

3.4 Helix VIII of BLT1 receptor

BLT1 belongs to the rhodopsin subfamily of GPCRs analysis of the crystal structure of rhodopsin confirmed the presence of 7 transmembrane (TM) helices and revealed the existence of an 8th helix (helix VIII) that projects at a right angle from the C terminus of TM7 (Palczewski, Kumasaka et al. 2000) . This short helix of rhodopsin is anchored by a palmitoyl group to the cytoplasmic leaflet of the cell membrane, and a hypothetical model has predicted that helix VIII interacts with the N-terminal helix of the Gα and Gγ subunits (Lu, Saldanha et al. 2002). BLT1 was demonstrated to contain the helix VIII in its intracellular C terminus (Okuno, Ago et al. 2003; Gaudreau, Beaulieu et al. 2004).

BLT1 mutants with a truncated or substituted helix VIII showed much higher LTB_4 binding than wild-type (WT) receptor in HEK293 and CHO cells, albeit with comparable expression on the cell surface (Okuno, Ago et al. 2003). Similar to the WT receptor, in mutant receptors LTB_4 promoted binding of GTPγS. However unlike WT-BLT1, the addition of GTPγS did not inhibit LTB_4 binding to the mutant receptors.

The mutant receptors maintained a high affinity for LTB_4, even in the presence of an excess amount of GTPγS. They also showed more prolonged intracellular signaling (*e.g.* calcium mobilization and metabolic activation) after LTB_4 treatment. The BLT1 model predicts a helix VIII extending from TM7, similar to the one observed in rhodopsin. The BLT1 model suggests that a pair of aromatic residues (Tyr-285 and Phe-300), which are positioned similarly to the conserved Tyr-306 and Phe-313 pair in rhodopsin, may stabilize the inactive form of the receptor by holding TM7 and helix VIII at almost a right angle to each other.

Gaudreau *et al.* (Gaudreau, Le Gouill et al. 2002) proposed that Thr-308 is involved in GRK6-mediated desensitization of BLT1 signaling. This suggested that Phosphorylation at that site may also play a role in inactivation of BLT1.

In addition Gaudreau *et al.* (Gaudreau, Beaulieu et al. 2004) also proposed that helix VIII is involved in a hydrophobic core containing other hydrophobic residues in helix 1 and disruption of this hydrophobic core may facilitate the irreversible activation of BLT1. Therefore Cell surface expression levels of helix VIII mutants of BLT2 are considerably reduced. The helix VIII of BLT2 might also be important for receptor sorting, Several studies suggest that helix VIII of BLT1 may play an important role in the inactivation of BLT1 after G-protein activation, possibly by sensing Gα subunits as being GTP-bound.

Using reconstitution of a budded baculovirus expression system, Masuda *et al.* (Masuda, Itoh et al. 2003) showed that trimeric G-proteins were required for maintenance of the high affinity state of BLT1. The binding affinity of GPCR is thus critically dependent on the status of G-proteins, which suggest that further studies will reveal an important role of helix VIII in downstream signaling.

4.0 Rho GTPases

Rho GTPases are members of the Ras superfamily of monomeric 20–30 kDa GTP-binding proteins. Ten different mammalian Rho GTPases, some with multiple isoforms, have been identified to date (Jones, Ridley et al. 2000). The most extensively characterized members are RhoA, Rac1 and 2 and Cdc42. Each of these GTPases act as a molecular switch, cycling between an active GTP-bound, and an inactive GDP-bound, state. In the GTP-bound form they are able to interact with effector or target molecules to initiate a downstream response, while an intrinsic GTPase activity returns the proteins to the GDP-bound state, to complete the cycle and terminate signal transduction.

The major function of Rho GTPases is to regulate the assembly and organization of the actin cytoskeleton (Hall 1998). The effects of 3 members of the Rho family RhoA, Rac and Cdc42 were initially described using quiescent Swiss3T3 fibroblasts, a cell line in which serum starvation creates a very low background of organized F-actin structures. Addition of lysophosphatidic acid induce the formation of contractile actin–myosin stress fibres and associated focal adhesions, and which was blocked by C3 transferase (Ridley and Hall 1992).

The dominant-negative Rac specifically inhibits the response (Ridley, Paterson et al. 1992).to Growth factors, such as platelet-derived growth factor, insulin or epidermal growth factor (EGF) which induce the formation of actin-rich lamellipodia and membrane ruffles associated with focal contacts.

Bradykinin a nine amino acid peptide induces the formation of peripheral microspikes or filopodia, which are also associated with focal contacts, and this can be inhibited by expression of dominant-negative Cdc42 (Kozma, Ahmed et al. 1995).

Rho GTPases have also been found to play a role in a variety of cellular processes that are dependent on the actin cytoskeleton, such as cytokinesis (Mabuchi, Hamaguchi et al. 1993; Prokopenko, Saint et al. 2000) phagocytosis (Cox, Chang et al. 1997; Caron and Hall 1998), pinocytosis (Ridley, Paterson et al. 1992), cell migration (Nobes and Hall 1999; Allen, Zicha et al. 1998), morphogenesis (Settleman 1999) and axon guidance (Luo, Jan et al. 1997).

In addition to their effect on the actin cytoskeleton, they also regulate a variety of other biochemical pathways, including serum response factor (SRF) and nuclear factor kB (NF-kB) transcription factors (Hill, Wynne et al. 1995; Perona, Montaner et al. 1997), the c-*jun* N-terminal kinase (JNK) and p38 mitogen-activated protein kinase pathways (Coso, Chiariello et al. 1995; Minden, Lin et al. 1995), the phagocytic NADPH oxidase complex (Abo, Pick et al. 1991), G_1 cell-cycle progression (Olson, Ashworth et al. 1995), the

assembly of cadherin-containing cell–cell contacts (Braga 1999), (Kaibuchi, Kuroda et al. 1999), secretion in mast cells (Norman, Price et al. 1996), cell polarity (Johnson 1999) and cell transformation (Van Aelst and D'Souza-Schorey 1997).

Therefore, although Rho GTPases are best characterized for their effects on the actin cytoskeleton, there is now much interest in their ability to affect cell proliferation and gene transcription and degranulation of neutrophils. Thus studies using inhibition always show some effect and are hard to analyse

5.0 Neutrophils Granules and Degranulation

5.1 Granule Population in Neutrophils.

Neutrophils contain at least four different types of granules identified by subcellular fractionation and transmission electron microscopy. These are: primary granules, also known as azurophilic granules, secondary granules, also known as specific granules, tertiary granules, and secretory vesicles. The primary granules are the main storage site of the most toxic neutrophil-derived mediators, including elastase, myeloperoxidase, cathepsins, and defensins. The secondary and tertiary granules contain lactoferrin and matrix metalloprotease-9 (MMP-9, also known as gelatinase B), respectively (Borregaard and Cowland 1997).

The secretory vesicles in human neutrophils contain human serum albumin, suggesting that they derive from endocytosis. The secondary and tertiary granules have similar contents but can be discriminated by their different intrinsic buoyant densities when centrifuged on gradient media (Kjeldsen, Sengelov et al. 1994). All of these granule types are retained in the cell cytoplasm and are not released until receptors in the plasma membrane or phagosomal membrane signal to the cytoplasm to activate their movement for secretion of their contents by exocytosis. Thus, the receptor-linked secretory pathway is an important control mechanism, as the neutrophil is highly enriched in tissue-destructive proteases and other enzymes.

5.2 Degranulation

Degranulation is defined as the secretion by receptor-mediated exocytosis of granule-derived substances. Upon receptor-mediated stimulation of neutrophils, granules are mobilized triggering their docking and content secretion which can occur intracellularly with microbe-laden phagosomes, or extracellularly at the plasma membrane. Upon exocytosis of granules, neutrophils release a diverse array of antimicrobial proteins and enzymes, many of which also possess tissue-damaging properties. At the same time, neutrophils release reactive oxygen species by respiratory burst, to aid in pathogen clearance, in addition to cytokines, which recruit additional leukocytes to the region of infection or inflammation.

A recent study by Brinkmann *et al* (Brinkmann, Reichard et al. 2004) described a novel mechanism causing elimination of microbial pathogens by neutrophils. Upon activation by ligands such as interleukin-8 (IL-8), lipopolysaccharide, and interferon-γ with complement 5a (Martinelli, Urosevic et al. 2004), neutrophils generated a web of extracellular fibres known as "neutrophil extracellular traps" (NETs), composed of DNA, histones, and antimicrobial granule proteins, which were highly effective at trapping and killing invasive microbes. The authors proposed that NETs amplified the effectiveness of antimicrobial components by concentrating them in a fibrous, net-like structure and reducing their exposure to host tissues.

Even though this report did not suggest the molecular mechanisms responsible for NET formation and its association with granular protein, it opened a new horizon in the field of neutrophil biology in relation to mediator release and bactericidal activity. Moreover, it has been recently suggested that NET formation may be an important mechanism for control of sepsis (Clark, Ma et al. 2007).

In many inflammatory disorders, such as acute lung injury, ischemia, reperfusion injury, severe asphyxic asthma, rheumatoid arthritis, and septic shock, excessive neutrophil degranulation is a common feature. Recent findings have identified a number of important signaling pathways in neutrophils that may be essential for neutrophil exocytosis.

5.3 Degranulation mechanism in neutrophils

Receptor stimulation of neutrophils by a secretagogue triggers granule translocation to the phagosomal or plasma membrane, where they dock and fuse, releasing their contents. The release of granule-derived mediators from neutrophils occurs by a tightly controlled receptor-coupled mechanism termed "regulated exocytosis". Exocytosis is postulated to take place in four discrete steps (Toonen and Verhage 2003).

The first step of exocytosis is granule recruitment from the cytoplasm and translocation to the target membrane, which is dependent on actin cytoskeleton remodeling and microtubule assembly (Burgoyne and Morgan 2003). This is followed by the second step of granule tethering and docking, leading to contact of the outer surface of the granule lipid bilayer membrane with the inner surface of the target membrane. Granule priming then follows as the third step to make granules fusion-competent and ensure that they fuse rapidly, to form a reversible pore structure between the granule and the target membrane.

The fourth and final step, granule fusion, occurs by rapid expansion of the fusion pore, leading to complete fusion of the granule membrane with the target membrane and expulsion of the granular contents to the outside of the cell.

This increases the total surface area of the cell, and exposes the interior surface of the granule membrane to the exterior. Tethering and docking steps require the sequential action of Rab and SNARE proteins (Stow, Manderson et al. 2006); whether these factors are also needed for subsequent steps of fusion remains to be determined.

Translocation and exocytosis of granules from neutrophils require, as a minimum, increases in intracellular Ca^{2+} and guanosine triphosphate (GTP) as well as hydrolysis of adenosine triphosphate (ATP) (Nusse and Lindau 1988; Theander, Lew et al. 2002).

Not surprisingly, there are numerous target molecules for these effectors, including Ca^{2+}-binding proteins such as annexins and calmodulin, and GTP-binding proteins such as heterotrimeric and small monomeric G proteins. The role of ATP in exocytosis as well as kinases, is to act as an energy source for SNARE complex reorganization and as a phosphate donor for phosphorylation of downstream effector molecules.

5.4 Actin cytoskeletal dynamics in exocytosis

Actin remodeling is clearly a prime downstream target of activated effector molecules during receptor-mediated exocytosis. The actin cytoskeleton forms a mesh around the periphery in many different kinds of secretory cells (i.e., neutrophils, mast cells, neurons and endocrine cells). This may act as a shield against aberrant granule docking and fusion at the plasma membrane. So in this case the actin cytoskeletal mesh would have to be disassembled during exocytosis (Norman, Price et al. 1994; Muallem, Kwiatkowska et al. 1995; Takao-Rikitsu, Mochida et al. 2004).

However, some studies suggest that F-actin has normally a very diffuse distribution in resting neutrophils, and only assembles into a cortical ring upon stimulation by f-Met-Leu-Phe (Downey, Elson et al. 1991; Filippi, Harris et al. 2004). In the latter studies, live

neutrophils were first adhered to poly-L-lysine-coated glass slides (which itself leads to activation), before stimulation with f-Met-Leu-Phe for a period of time prior to fixation. Therefore, this procedure may lead to F-actin remodeling prior to stimulation.

Several other studies report that actin remodelling is needed to direct neutrophil migratory responses (Affolter and Weijer 2005). Directed neutrophil movements, or chemotaxis towards sites of infection, are driven by polarized F-actin formation, with the leading edge of cells showing enhanced levels of actin polymerization. Interestingly, neutrophils produce a polarized response even when subjected to uniform concentrations of chemoattractants, and maintain this polarity via continued production of activated effector molecules such as 3-phosphoinositol lipids in the cell membrane (Zigmond, Levitsky et al. 1981; Xu, Wang et al. 2003). A recent comprehensive study of neutrophil granule proteins revealed that actin associates with all granule subsets, which suggests that actin may play an active role in regulating neutrophil exocytosis (Jog, Rane et al. 2007).

5.5 Ca^{2+} signaling in exocytosis

Increases in intracellular Ca^{2+} alone are sufficient to induce the release of granules from neutrophils, particularly if the concentration of Ca^{2+} is elevated to sufficiently high levels by the use of Ca^{2+} ionophores such as A23187 or ionomycin. A hierarchy of granule release exists in response to elevating concentrations of Ca^{2+} (Pinxteren, O'Sullivan et al. 2000; Sengelov, Kjeldsen et al. 1993). The order of release is secretory vesicles > tertiary granules > secondary granules > primary granules (Sengelov, Kjeldsen et al. 1993; Bentwood and Henson 1980).

The release of each type of granule appears to be differentially regulated by unique intracellular signaling pathways. Activation of many neutrophil receptors causes elevated Ca^{2+} levels, including the seven transmembrane-spanning G protein-coupled receptors such as the formyl peptide receptor (that binds the bacterial tripeptide, f-Met-Leu-Phe) and chemokine receptors (such as the interleukin-8 receptor, CXCR1) (Andersson, Dahlgren et al. 1986; Itagaki, Kannan et al. 2002). Although Ca^{2+} is a crucial second messenger in the activation of exocytosis, the specific target molecules for Ca^{2+} in neutrophil degranulation have not yet been identified. Several candidates have been suggested, notably annexins, protein kinase C, and calmodulin, all of which bind Ca^{2+} to modulate their activities (Brown, Reed et al. 1991; Sjolin, Stendahl et al. 1994; Steadman, Petersen et al. 1996; Donnelly and Moss 1997; Haribabu, Richardson et al. 2000). Neutrophils have been shown to require these Ca^{2+}-binding proteins for granuletranslocation and exocytosis.

5.6 Phospholipid signaling in degranulation

Numerous studies have indicated a role for phospholipids, particularly polyphosphoinositides, in regulation of neutrophil degranulation. Polyphosphoinositide production, such as phosphatidylinositol 4,5-bisphosphate (PIP_2), induced by the activation of the hematopoietic cell-specific isoform phosphatidylinositol 3-kinase-g (PI3Kg) has been shown to be required for granule exocytosis in permeabilized neutrophilic-like cells, HL-60 cells (Fensome, Cunningham et al. 1996).

Intracellular sites of PIP_2 formation in neutrophils are not known, but are likely to occur both at the plasma and granule membranes. Regions of PIP_2 enrichment in the membrane form essential binding sites for many intracellular signaling molecules, particularly those that contain pleckstrin homology domains, such as Rho guanine exchange factors including Vav, which signal downstream to Rho guanosine triphosphatases (GTPases) (Kim, Marchal et al. 2003; Gakidis, Cullere et al. 2004; Fumagalli, Zhang et al. 2007) .

Phosphatidylinositol transfer protein (PI-TP) has been shown to be essential for the transport of phosphatidylinositol to cellular membranes as a substrate for PI3K activity to generate PIP_2, and is also capable of restoring exocytotic responses in HL-60 cells (Fensome, Cunningham et al. 1996).

In addition, a role for phospholipase D has been indicated in neutrophil degranulation, particularly for primary and secondary granule release, as its product, phosphatidic acid, induces the release of these granules (Kaldi, Szeberenyi et al. 2002). Phosphatidic acid and its lipid derivative, lysophosphatidic acid, may act as second messengers for downstream reactions, but they clearly aid fusion through the generation of membrane curvature (Kooijman, Chupin et al. 2003). Thus, membrane lipid remodelling is also an essential component of degranulation in neutrophils.

5.7 Role for src family kinases in neutrophil degranulation

Protein phosphorylation is a critical event in neutrophil activation leading from receptor stimulation to exocytosis. Phosphorylation is carried out by kinases which are themselves frequently activated by phosphorylation of molecules situated upstream. This specifically involves the attachment of a phosphate molecule, donated by intracellular ATP, to a key site in the effector molecule, leading to conformational changes that cause activation. Receptor stimulation through the formyl peptide receptor by f-Met-Leu-Phe leads to phosphorylation of a wide range of kinases which then activate their respective effector pathways.

Protein kinases can be discriminated based on their affinity for different amino acid residues in effector molecules. Thus, serine/threonine kinases and tyrosine kinases have been characterized as two distinct types of kinases involved in receptor signaling. Tyrosine kinases are further differentiated for their intrinsic association with the intracellular domain of receptors (receptor tyrosine kinases) or as cytosolic enzymes (nonreceptor tyrosine kinases). The *src* family of nonreceptor tyrosine kinases have been implicated in the control of exocytosis of granule products from neutrophils.

Three *src* family members, Hck, Fgr, and Lyn, are expressed in neutrophils and activated by f-Met-Leu-Phe receptor stimulation. Hck translocates to the myeloperoxidase-positive primary granule population following cell activation (Mohn, Le Cabec et al. 1995;

Kooijman, Chupin et al. 2003; Gutkind and Robbins 1989) , while Fgr becomes associated with the lactoferrin-containing secondary granules during exocytosis (Mocsai, Jakus et al. 2000). The selective recruitment of *src* kinases indicates that different signaling pathways exist in neutrophils to induce the release of each granule population. Treatment of human neutrophils with the *src* family inhibitor PP1 has led to inhibition of the release of primary granules, secondary granules, and secretory vesicles in response to f-Met-Leu-Phe (Barlic, Andrews et al. 2000). Neutrophils isolated from $hck^{-/-}fgr^{-/-}lyn^{-/-}$ triple knockout mice also showed a deficiency in secondary granule release of lactoferrin, although it was not possible to determine primary granule release of glucuronidase from murine cells(Barlic, Andrews et al. 2000) .

Hck and Fgr were recently shown to regulate the activation of the Rho GEF and Vav1 that signals through the Rho GTPase and Rac inducing actin polymerization and superoxide release (Fumagalli, Zhang et al. 2007). The deficiency in secondary granule release in triple knockout neutrophils correlated with reduced p38 mitogen-activated protein (MAP) kinase activity, suggesting that *src* kinases act upstream of p38 MAP kinase. Indeed, treatment of human neutrophils with the p38 MAP kinase inhibitor, SB203580, led to reduced primary and secondary granule exocytosis in response to fMLP (Barlic, Andrews et al. 2000).

However, another MAP kinase inhibitor, PD98059, which blocks ERK1/2 activity, did not affect release of primary and secondary granules or secretory vesicles (Barlic, Andrews et al. 2000). These findings indicate that *src* kinases, Hck, Fgr, and Lyn, along with p38 MAP kinase, but not ERK1/2, play a role in regulating the release of granules.

Gaudreault et al. (Gaudreault, Thompson et al. 2005) demonstrated that there is partial involvement of src family kinase potentially Yes kinase in LTB_4 mediated neutrophil degranulation and that receptor endocytosis is crucial for Yes kinase activation.

5.8 Arrestin function in regulating exocytosis

A group of scaffolding proteins known as arrestins 2 and 3 have also been shown to be required for activating signaling pathways leading to exocytosis of primary and secondary granules in neutrophils (Ferguson, Downey et al. 1996; Barlic, Andrews et al. 2000). Arrestins 2 and 3 are cytosolic phosphoproteins that were previously characterized for their role in endocytosis of ligand-bound chemokine receptors, particularly CXCR1, which is the high affinity receptor for the neutrophil chemotactic factor, IL-8. Arrestins 2 and 3 act by uncoupling activated G protein-coupled receptors from their associated heterotrimeric G proteins, they bind directly to the cytoplasmic tail of the CXCR1 receptor (Barlic, Andrews et al. 2000; Ferguson, Downey et al. 1996).

Dominant negative mutants of arrestin 2 and 3 were shown to inhibit the release of granules when transfected into rat basophilic leukemia (RBL) cells, a cell line resembling mast cells and basophils (Barlic, Andrews et al. 2000). Interestingly, arrestins 2 and 3 also associate with the primary and secondary granules in IL-8-activated neutrophils, and they do so by binding to Hck and Fgr, respectively (Barlic, Andrews et al. 2000). Thus, arrestins 2 and 3 act at two sites in the cell during chemokine activation; one at the receptor in the plasma membrane and a second on granule membranes.

Hypothesis

The Role of helix VIII of BLT1 in neutrophil responses to LTB_4 is well documented (Shimizu, Yokomizo et al. 2000; Okuno, Ago et al. 2003; Gaudreau, Beaulieu et al. 2004). Our lab and others have shown that truncation or mutation in BLT1 can lead to abnormal signaling in neutrophils (Baneres, Martin et al. 2003; Gaudreau, Beaulieu et al. 2004; Okuno, Yokomizo et al. 2005). Gaudreau et al. (Gaudreau, Beaulieu et al. 2004) have demonstrated that a mutation in helix VIII leads to significant reduction in BLT1 internalization. Gaudreault, et al. have shown that receptor endocytosis is crucial for LTB_4-mediated neutrophil degranulation, whereas Lambert et al. demonstrated that a defect in helix VIII of BLT1 leads to multiple pseudopod formation and defective chemotaxis. They also proposed that a member of the Rho family of GTPases, RhoA, is important for LTB_4-mediated chemotaxis.

Since the intracellular granule movement and granule exocytosis in neutrophils require cytoskeleton rearrangement in a similar fashion as in chemotaxis which has been shown to be RhoA-dependent and receptor endocytosis is crucial for LTB4-mediated degranulation, we hypothesised that the dileucine motif in helix VIII of BLT1 receptor plays a role in RhoA-dependent neutrophil degranulation.

Objectives

To study the role of the dileucine motif and RhoA in neutrophil degranulation.

- To test the PLB985 cell line stably transfected with BLT1 (wt) or BLT1 (mutant) as a model for LTB4 -induced neutrophil degranulation.
- To determine whether the 2LA mutation in BLT1 effects LTB4-mediated degranulation.
- To determine the signaling pathways involved in wt and mutant BLT1 receptor-mediated degranulation using pharmacological inhibitors.
- To determine whether the defective signaling in mutant BLT1 is reversible by co-expression of constitutively active RhoA.

Materials and Methods

Reagents

RPMI 1640 and geneticin (G418) were from Invitrogen Canada. FBS, cytochalasin B, Y-27632 and Triton were from Sigma-Aldrich Canada. DMSO was from Fischer Scientific.

Dextran and Ficoll-Paque PLUS, were from Amersham Biosciences. U75302,4-amino-5-(4methylphenyl)-7-(*tert*-butyl)pyrazolo[3,4-*d*] pyrimidine (PP1), *N*-benzyl-3,4-dihydroxylbenzylidenecyanoacetamide (AG490), 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB203580), 2'-amino-3'-methoxyflavone (PD98059), , LY294002, and *p*-nitrophenyl *N*-acetyl-β-glucosaminide,Wortmannin and Pertussis toxin were from BIOMOL. LTB_4 was from Cayman Chemicals. BSA was from BIO MEDIA Canada.

Isolation of human neutrophils

Neutrophils were obtained from peripheral blood of healthy medication-free volunteers after informed consent in accordance with an Internal Review Board-approved protocol, as described previously (Stankova, Turcotte et al. 2002). Briefly, peripheral blood leukocytes were enriched by dextran sedimentation, layered over a Ficoll-Hypaque cushion, and centrifuged at 4000 x *g* for 20 min. mononuclear leukocytes were collected at the interface, whereas neutrophils were obtained from the pellet. The neutrophils preparation was depleted of erythrocytes by osmotic shock, then washed and resuspended in PBS until used.

β-Hexosaminidase release assays

Degranulation was determined by measuring the release of a granule marker, β-hexosaminidase, as described previously by Ali et al. (Ali, Richardson et al. 1993), with some modifications. For PLB-BLT cells, after 3 days of differentiation, 2.5 x 10^5 cells were washed once with 1 ml of PBS. For human neutrophils, 4 x 10^6 cells were used. When necessary, cells were incubated with inhibitors for indicated times at 37°C. Cell pellets were resuspended in 250 µl of PBS containing cytochalasin B (4.8 µg/ml) and incubated for 5 min. Cytochalasin B was used to facilitate degranulation without priming

cells with other reagents that might interfere directly in intracellular signaling pathways. Cells were then stimulated with LTB_4 (100 nM) or other stimuli for 10 min.

After stimulation, β-hexosaminidase activity was measured in 50 µl of cell-free supernatant by spectrophotometric analysis using 50µl of 2 mM *p*-nitrophenyl-*N*-acetyl-β-glucosaminide as chromogenic substrate. Cell supernatant and substrate were incubated for 1 h at 37°C. The reaction was stopped by adding 150 µl of a 0.1M Na_2CO_3-$NaHCO_3$ buffer at pH 9.5. OD was then read at 405 nm using a spectrophotometer (BioRad). Values were expressed as percentages of total β-hexosaminidase, which was determined in cells lysed with 0.1% Triton X-100. All percentages were corrected by subtracting spontaneous β-hexosaminidase release in cell supernatants. All assays were performed in triplicate, and OD was read three times.

Construction of Myc-tagged wild type and mutant receptors.

The cloning of WT BLT1-cDNA and generation of the mutant 2L (304-305)A in which leucines were substituted with alanines was previously described (Gaudreau, Le Gouill et al. 1998; Gaudreau, Beaulieu et al. 2004). All constructions were subcloned into $pcDNA_3$ expression vector (invitrogen) and sequenced (University of Calgary, Alberta, Canada).

Cell culture and transfection

PLB-985 cells were grown in RPMI 1640 supplemented with 5% FBS and gentamicin sulfate (40 μg/ml). All cells were cultured at 37°C in a humidified 5% CO_2 incubator.

PLB-985 cells were stably transfected with a pcDNA3 vector containing a construct encoding for a myc-tagged BLT1 sequence (Gaudreau, Le Gouill et al. 2002) or myc-tagged BLT1 mutant sequence (Gaudreau, Beaulieu et al. 2004) . In short, 30 x 10^6 cells were electroporated at 320 V using 30 μg of pcDNA3-BLT. Cells were then cultured for 3 wk in medium containing G418 at a concentration of 800 μg/ml. After 2 wk of G418 selection, cells were sorted twice using a FACSVantage cell sorter (BD Biosciences). These cells are referred to as PLB-BLT1 (WT) and PLB- BLT1 (2LA).

Differentiated PLB-BLT1 (wt) cells or PLB-BLT1 (2LA) (10 x 10^6 cells), loaded in nucleofection buffer, were nucleofected with 10 μg of plasmid containing constitutively active RhoA or an empty vector pcDNA3 using nucleofector program *U02* (Amaxa Biosystems). For degranulation experiments, cells were used 8 h post transfection PLB-BLT1 (WT) and PLB- BLT1 (2LA) cells were cultured in medium supplemented with 1.25% DMSO for 3 days before each experiment to induce cell differentiation into a neutrophil-like phenotype, unless mentioned otherwise.

Statistical analyses

Data were analyzed by using PRISM5 software. Paired Student's t test was performed where indicated. Differences were considered significant at $p \leq 0.05$ for $n \geq 3$.

Results

The Dileucine motif in helixVIII of the BLT1 receptor plays an important role in LTB4 mediated degranulation in neutrophils.

We and others (Yokomizo, Masuda et al. 2000; Okuno, Ago et al. 2003; Gaudreau, Beaulieu et al. 2004; Okuno, Yokomizo et al. 2005) have shown that the distal 2LA (304-305) motif, which is in helix VIII of BLT1 receptor plays an important role in phospholipase C activation, receptor desensitization, internalization and chemotaxis. To investigate whether mutation of dileucine motifs of BLT1 affects degranulation, we used PLB-985 cells stably transfected with BLT1-wt and the mutant 2LA (304-305). Those cells were cultured in medium supplemented with DMSO for 3 days to induce cell differentiation into a neutrophil-like phenotype. Differentiated PLB-BLT1 (wt) cells and freshly acquired human neutrophils were stimulated with LTB_4, its respective vehicle ethanol and fMLP as a control. LTB_4 mediated degranulation levels in PLB-BLT1 (wt) (fig1A) and freshly acquired human neutrophils (fig1B) were comparable. The degranulation levels in PLB-BLT1 (wt) (fig1C) and human neutrophils (fig1D) were concentration dependent. Degranulation levels in PLB-BLT1 (2LA) mutant cells were 60±5% less (fig2B) compared to PLB-BLT1 (wt) cells (fig2A) and human neutrophils (fig 1B). We also observed that PLB-BLT1 (2LA) did not degranulate in a concentration

dependent manner. In contrast to PLB-BLT1 (wt) cells and neutrophils, increasing the concentration of LTB4 failed to bring about any change in degranulation levels of PLB-BLT1 (2LA) cells (fig 2C). This strongly suggests that the distal dileucine motif in helix VIII plays a crucial part in LTB_4 mediated downwards signaling in neutrophil degranulation. It was interesting to see that there remained a residual LTB_4 dependent 35-40% degranulation in PLB cells transfected with 2LA.

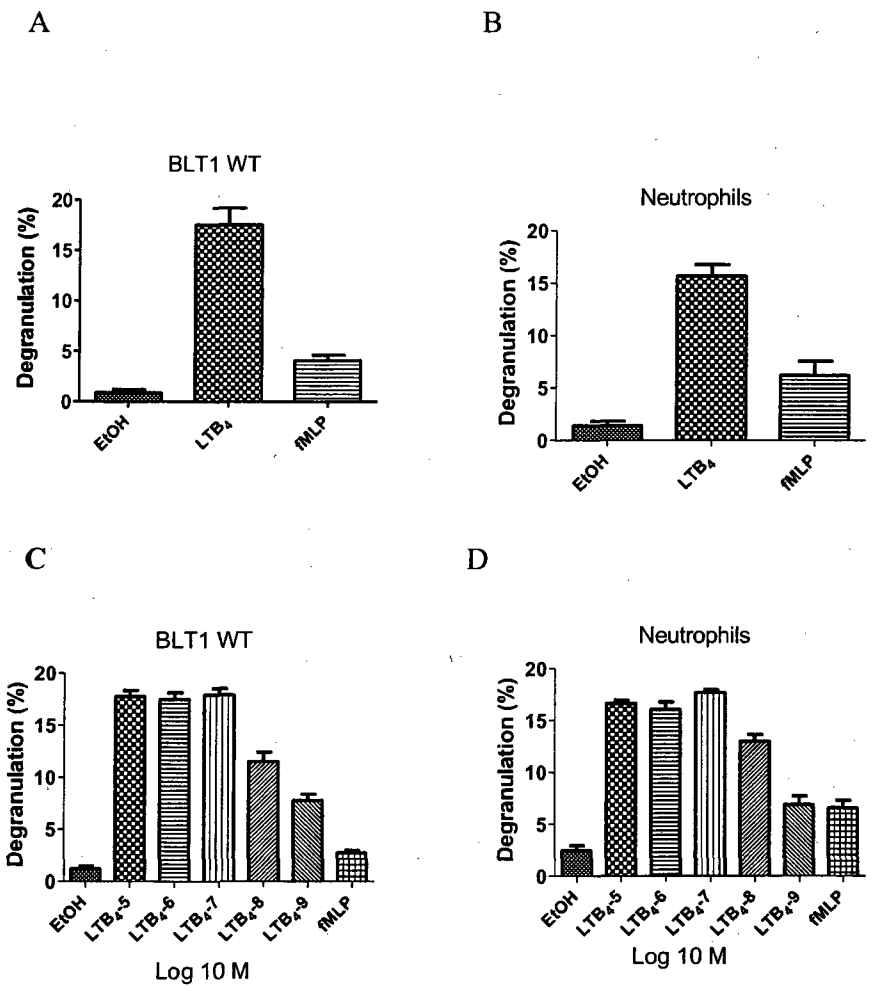


Fig 1: PLBB-BLT1 (WT) and Neutrophil degranulation.

A) PLB-P85 cells stably transfected with BLT 1 (WT) differentiated for 0-3 days using DMSO. (2.5×10^5) and, **B)** freshly isolated human neutrophils (3×10^{-6}) were stimulated with 10^{-7}M of LTB4 or its appropriate vehicle (EtOH) or 10^{-7}M fMLP (positive control) for 10 min. After stimulation, β-hexosaminidase release was assessed. **C)** PLB-P85 cells stably transfected with BLT1 (WT) and **D)** Freshly isolated human neutrophils (3×10^6) were stimulated with of LTB4 (10^{-5}- 10^{-9}M) or its appropriate vehicle (ethanol EtOH) or 10^{-7}M fMLP (positive control) for 10 min. After stimulation, β-hexosaminidase release was assessed

n= 3

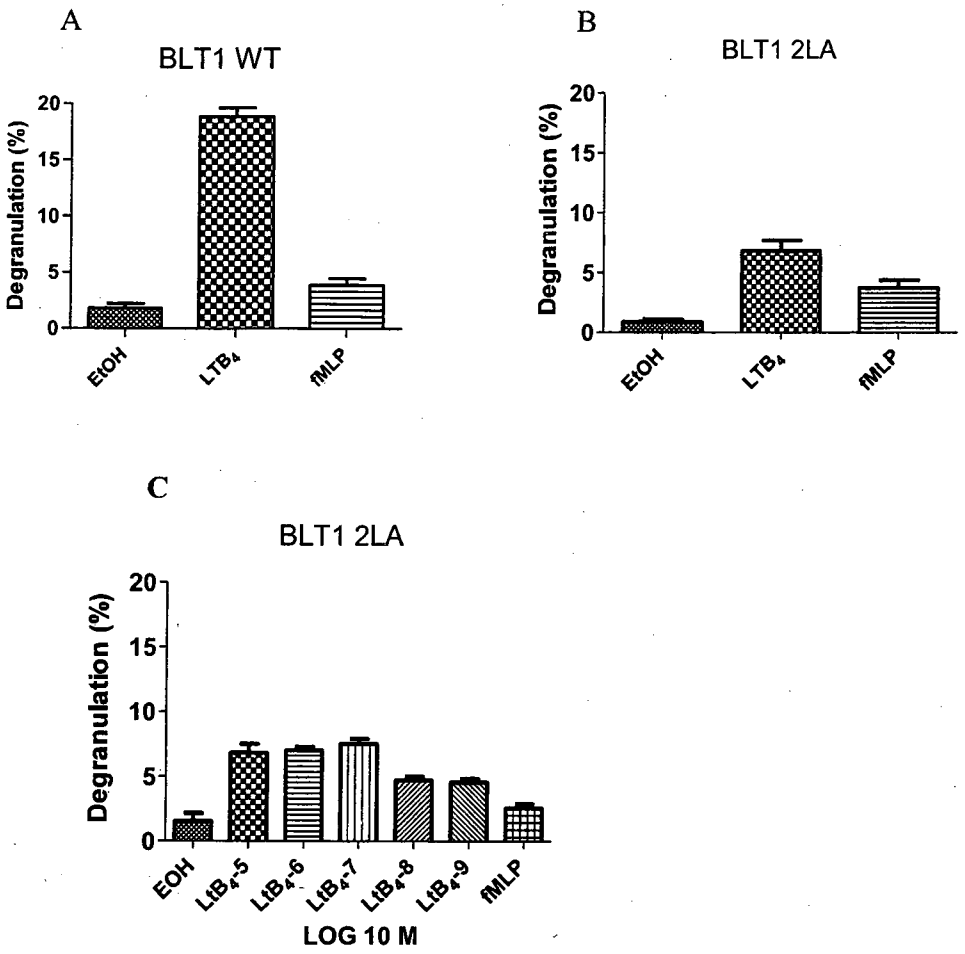


FIG 2: PLB BLT1 (2LA) and PLB BLT1 (WT) degranulation

A) PLB-P85 cells stably transfected with BLT1 (WT) and, **B)** PLB-985 cells stably transfected with BLT 1 (2LA) (2.5×10^5) were differentiated for 0-3 days using DMSO and were stimulated with 10^{-7}M of LTB4 or it appropriate vehicle (EtOH) or 10^{-7}M fMLP (positive control) for 10 min. After stimulation, β-hexosaminidase release was assessed. **C)** PLB-P85 cells stably transfected with BLT1 (2LA) were differentiated for 0-3 days using DMSO (2.5×10^5) were stimulated with (10^{-5}- 10^{-9}M) of LTB4 or its appropriate vehicle (EtOH) or 10^{-7}M fMLP (positive control) for 10 min. After stimulation, β-hexosaminidase release was assessed.

n= 3

A Pertussis –sensitive Gαi protein subunit, PI3k and Src family kinases are involved in LTB4 mediated neutrophil degranulation.

Signaling of BLT1 through Gαi protein subunit and its involvement in neutrophils degranulation has been shown previously (Masuda, Itoh et al. 2003; Gaudreault, Thompson et al. 2005). In order to assess whether the residual degranulation in PLB-BLT1 (2LA) cells was also dependent on Gαi protein subunit signaling, we used pertussis toxin, an inhibitor of Gαi and Gα0 signaling.

PLB-BLT1 (wt), PLB-BLT1 (2LA) and human neutrophils cell were pretreated with PTX (50nM) for 6 h, followed by 10 min stimulation with LTB4 (100nM). Pretreatment with PTX resulted in 80 % reduction in degranulation levels of PLB-BLT1 (wt) (fig3A) and 40% reduction in degranulation levels of neutrophils (fig3C), while PTX pretreatment did not have any significant effect on PLB-BLT1 (2LA) residual degranulation (fig 3B). Previously it has been shown that BLT-1 mediated degranulation in RBL-2H3 cells (Ito, Yokomizo et al. 2002) and PLB985 cells (Gaudreault, Thompson et al. 2005) stably transfected with BLT1 cDNA was partially dependent on PI3k. To see if the residual degranulation in PLB-BLT1 (2LA) cells was dependent on PI3K activation, we used the specific PI3k inhibitor wortmanin (1μM).

Pre-incubation for 1h with wortmanin followed by stimulation with LTB_4 led to 60% inhibition of degranulation of PLB-BLT1 (wt) (fig4A) and 60% inhibition of human neutrophils (fig4C) degranulation. Incubation with wortmanin did not produce any reduction in degranulation levels in PLB-BLT1 (2LA) cells (fig4B).

Gαi protein subunit activation is known to play an important role in activation of several kinases including src kinase by different GPCR. Src family kinases are also known to be involved in certain neutrophil functions including granule secretion (Gaudreault, Thompson et al. 2005). To assess whether the residual degranulation in PLB-BLT1 (2LA) is dependent on scr kinase activation, PLB-BLT1wt and PLB-BLT12LA cells were pre-incubated or not with src kinase inhibitor PP1 (10μM) for 1h and then stimulated with LTB_4. PLB-BLT1 (wt) (fig5A) cells showed 40% reduction of degranulation levels compared to non pre-incubated cells, whereas PLB-BLT1 (2LA) (fig5B) cells did not show a significant reduction in degranulation levels. Human neutrophils pre-incubated with the inhibitor pp1 (30 μM) for 1h showed 45% reduction of degranulation levels after stimulation with LTB_4 (fig5C). Moreover PPI inhibition of PLB-BLT1 (wt) was concentration dependent, reaching 65% at the inhibitor concentration of 50μM (fig5D). There was no effect of increased concentration on PLB-BLT1 (2LA) mutant cells. The degranulation rate remained at similar level from 0-50μM (fig5E).

These results indicate that the residual degranulation by 2LA mutant BLT1 is not the result of downstream signaling of $G\alpha_{i/o}$, PI3K, or src kinase activation.

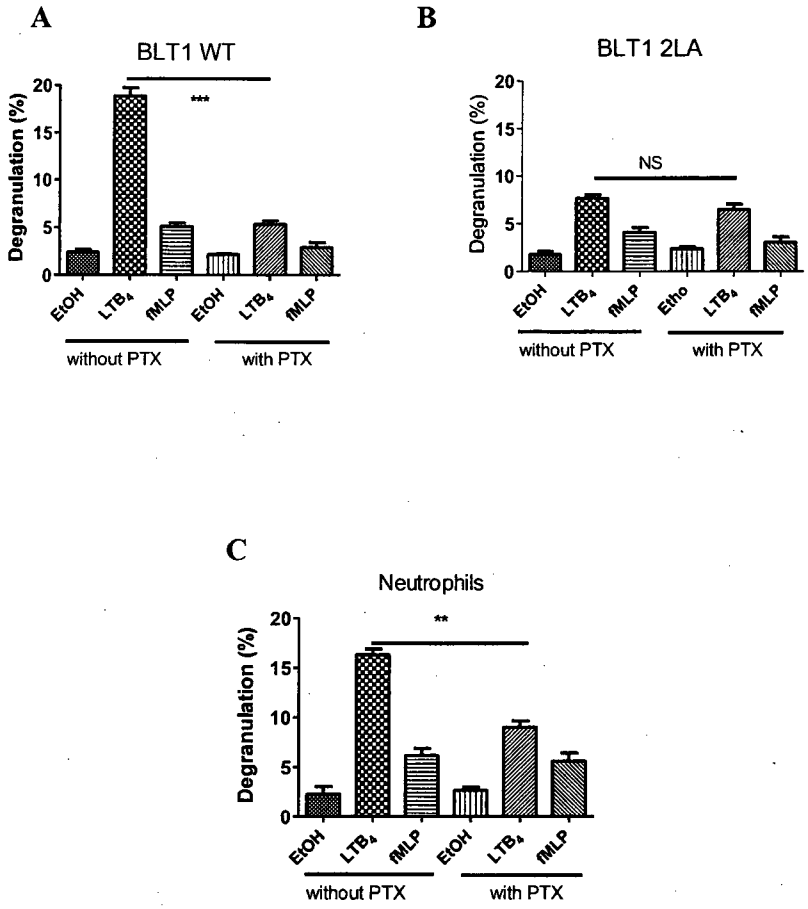


Fig 3: Effects of $G\alpha_i$ protein subunit blockade on LTB_4-mediated degranulation.

A) PLB-BLT1 (WT) cells, B) PLB-BLT1 (2LA) and C) Human neutrophils were pretreated or not with *Bordetella pertussis* toxin (50 ng/ml) at 37°C for 6 h. After incubation with PTX, cells were stimulated with 100 nM LTB_4 or its appropriate vehicle (ethanol; EtOH) or fMLP for 10 min. After stimulation, β-hexosaminidase release was assessed.

n= 3

***=P<0.0002 **=P<0.001

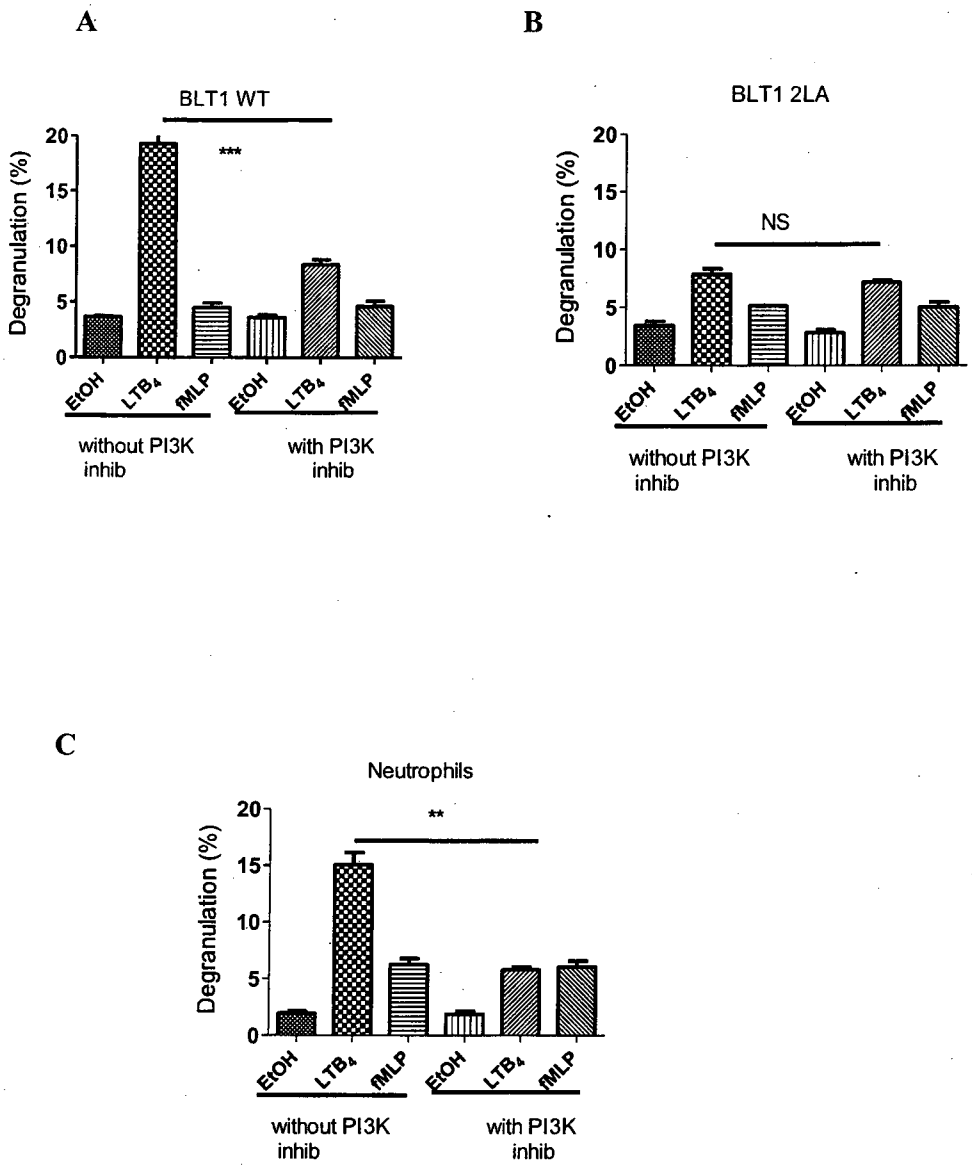


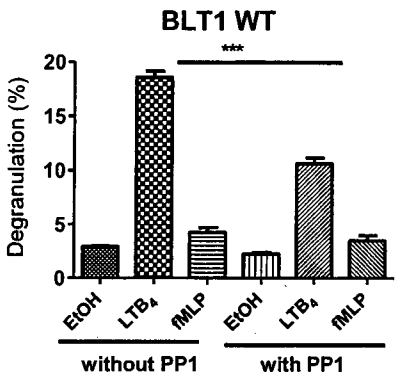
Fig 4. Effects of PI3K inhibition on LTB_4-mediated degranulation.

A) PLB-BLT1 (WT) cells, **B)** PLB-BLT1 (2LA) and **C)** human neutrophils were pretreated or not with wortmannin (1 μM) at 37°C for 1 h. After incubation with the inhibitor, cells were stimulated with 100 nM of LTB_4, its appropriate vehicle (ethanol) or fMLP for 10 min. After stimulation, β-hexosaminidase release was assessed.

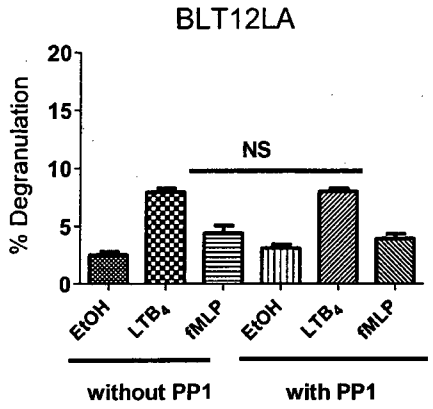
n= 3

***=P<0.0004, **=P<0.001

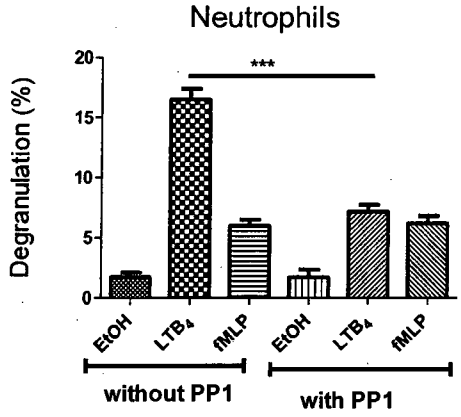
A


BLT1 WT
***
Degranulation (%)
20
15
10
5
0
EtOH
LTB4
fMLP
EtOH
LTB4
fMLP
without PP1
with PP1

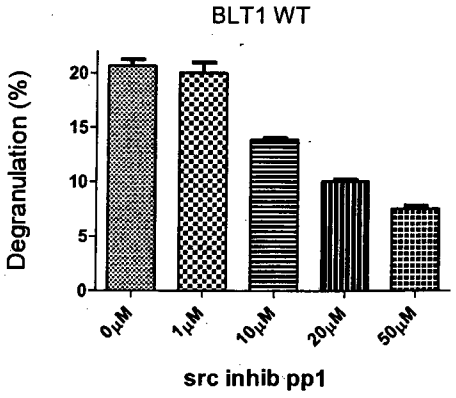
B


BLT12LA
% Degranulation
NS
20
15
10
5
0
EtOH
LTB4
fMLP
EtOH
LTB4
fMLP
without PP1
with PP1

C


Neutrophils
***
Degranulation (%)
20
15
10
5
0
EtOH
LTB4
fMLP
EtOH
LTB4
fMLP
without PP1
with PP1

D


BLT1 WT
Degranulation (%)
20
15
10
5
0
0μM
1μM
10μM
20μM
50μM
src inhib pp1

E

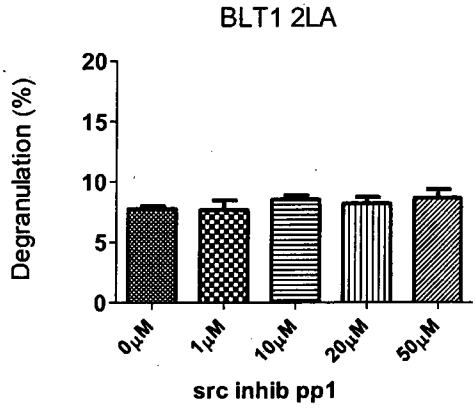

BLT1 2LA
Degranulation (%)
20
15
10
5
0
0μM
1μM
10μM
20μM
50μM
src inhib pp1

Fig5: Effects of inhibition of the Src family of the kinase on LTB_4-mediated degranulation in PLB-BLT cells and human neutrophils.

A) PLB-BLT1 (wt) cells (2.5×10^5) and **B)** PLB-BLT1 (2LA) cells (2.5×10^5) were pretreated or not with Src family kinases inhibitor PP1 (10 μM) for 1 h at 37°C and were then stimulated with LTB_4 (100 nM) or its vehicle (ethanol; EtOH) or fMLP for 10 min. After stimulation, β-hexosaminidase release was assessed. **C)** Human neutrophils (3×10^6 cells), isolated from peripheral blood were pretreated or not with PP1 (30 μM) for 1h at 37°C and were then stimulated with LTB_4 (100 nM) or its vehicle (ethanol) or fMLP for 10 min. After stimulation, β-hexosaminidase release was assessed. **D)** Concentration-dependent inhibition of PLB-BLT1 (wt) degranulation using PP1 (0–50 μM). **E)** Concentration-dependent inhibition of PLB-BLT1 (2LA) degranulation using PP1 (0–50 μM).

n= ≥3

***=P<0.0001

**=P<0.0009

JAK2, P38 kinase and MEK/ERK kinases do not play a role in BLT1 mediated degranulation.

Leukotrienes have been shown to activate JAK/STAT pathways (Lukashova, Chen et al. 2003) as well as MAPK signaling pathways (Mocsai, Jakus et al. 2000; Petrin, Turcotte et al. 2006).

To determine whether residual LTB_4-dependent degranulation in PLB-BLT1 (2LA) cells was mediated by JAK/STAT or MAPK signaling pathways we used specific inhibitors for JAK2, P38 kinase, JNK and MEK/ERK kinases.

Human neutrophils, PLB-BLT1 (wt) and PLB-BLT1 (2LA) were pre-incubated with P38kinase inhibitor SB203580 (10μM) for 1h. Our results indicated that in human neutrophils there was a statistically significant reduction of degranulation levels (35%) (fig6C). PLB-BLT1 (wt) (fig6A) and PLB-BLT1 (2LA) (fig6B) did not show significant reduction in degranulation levels.

PLB-BLT1 (wt), PLB-BLT (2LA) and human neutrophils were pre-treated with MEK/ERK kinase inhibitor PD980059 (10μM) (fig7.A-C) or JNK inhibitor (SP600125) (fig8.A-C) or JAK2 inhibitor, AG490 (10μM) (fig9.A-C) for 1h. No statistically significant reduction in degranulation was observed compared to PLB-BLT1 (wt), PLB-BLT1 (2LA) and human neutrophils stimulated with LTB_4 alone.

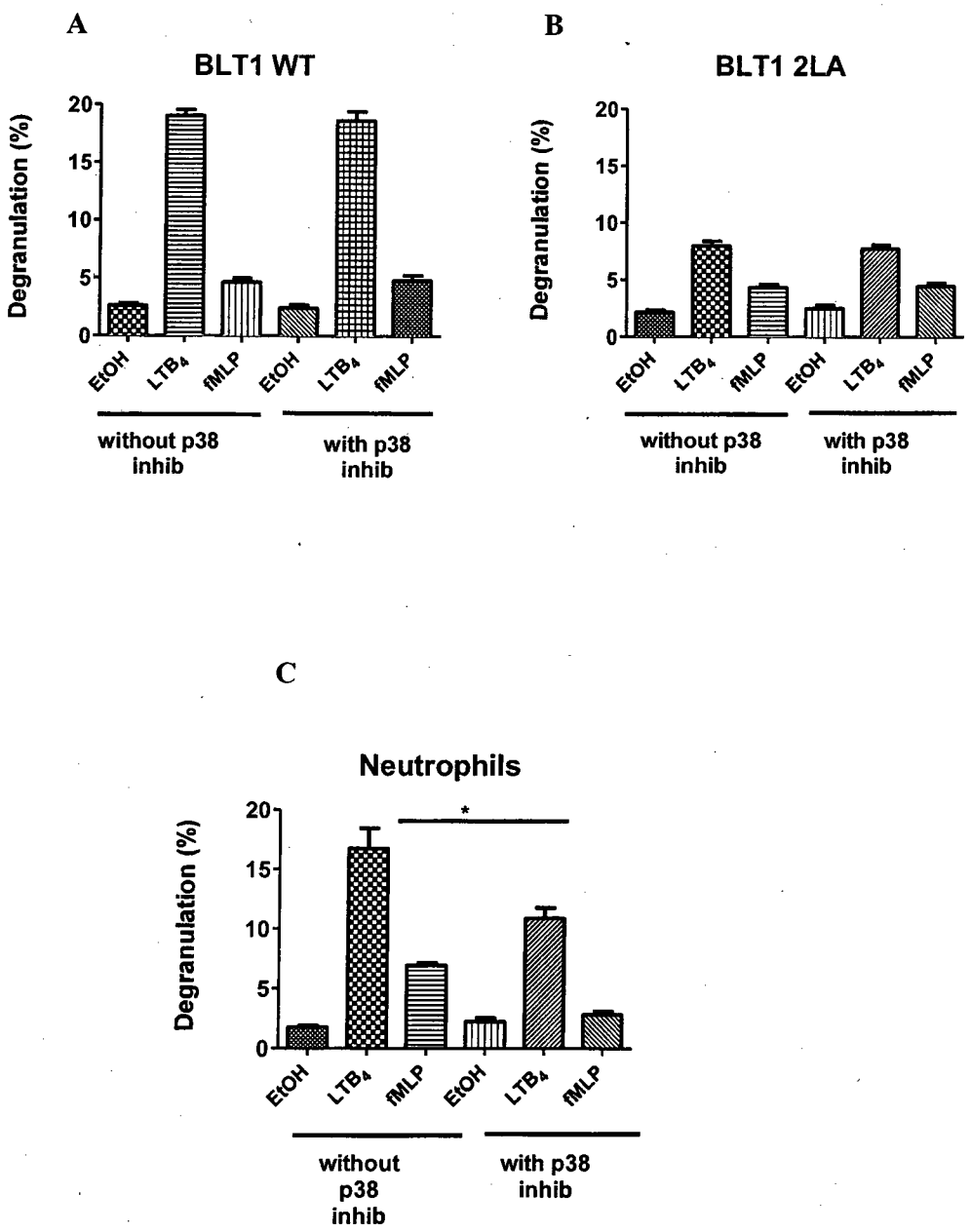


FIG 6: Effects of, p38 inhibition on LTB_4-mediated degranulation of PLB-BLT1 (wt), PLB-BLT1 (2LA) cells and human neutrophils

A) PLB-BLT (WT) cells (2.5×10^5 cells), B) PLB-BLT (2LA) cells (2.5×10^5 cells) and C) human neutrophils were pre-treated or not with p38 kinase inhibitor (SB203580), 10 or 30 μM) for 1 h at 37°C followed by stimulation with LTB_4 (100 nM), its vehicle (ethanol; EtOH) or fMLP for 10 min After stimulation, β-hexosaminidase release was assessed.

n= 3.*=P<0.05

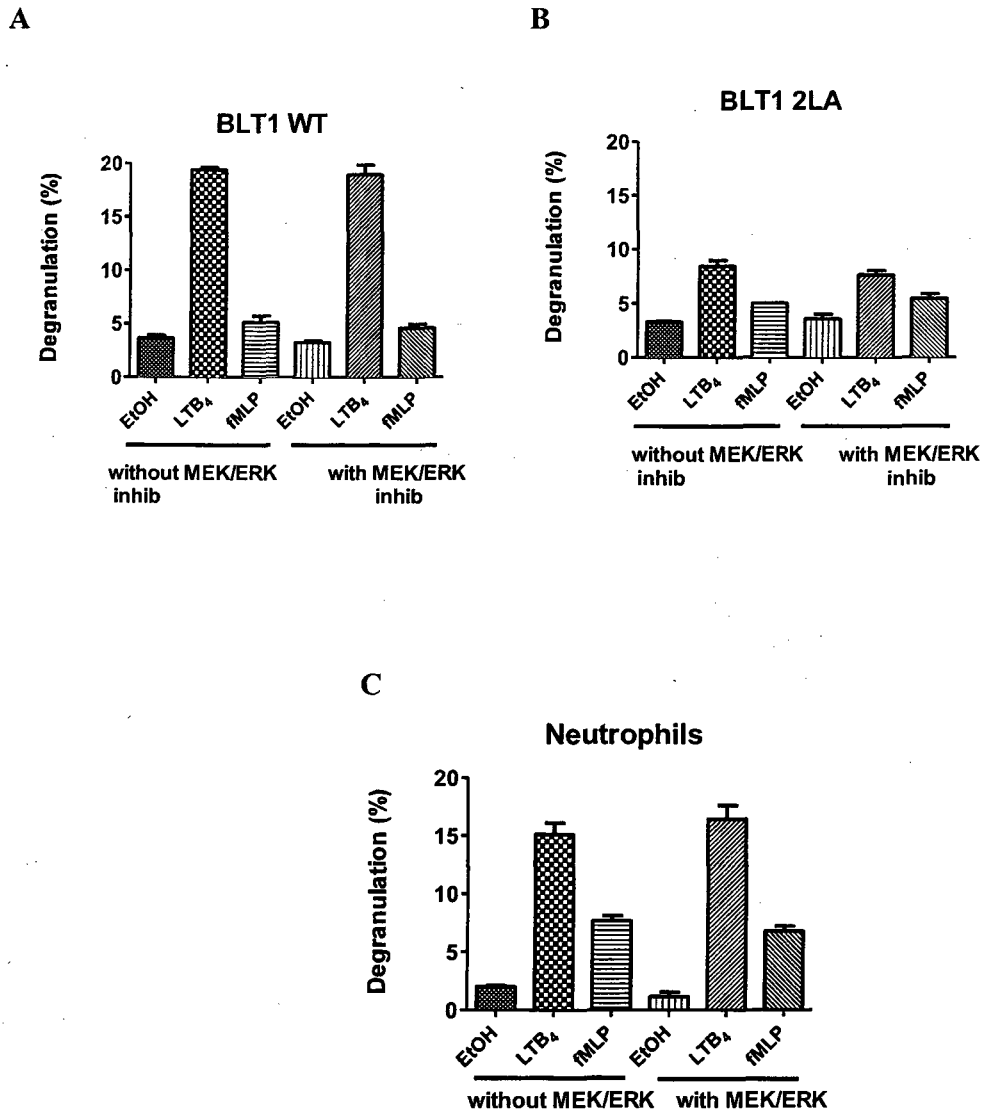


FIG 7: Effects of MEK/ERK inhibition on LTB_4-mediated degranulation of PLB-BLT1 (wt), PLB-BLT1 (2LA) cells and human neutrophils

A) PLB-BLT (WT) cells (2.5 x 10^5 cells), B) PLB-BLT (2LA) cells (2.5 x 10^5 cells) and C) human neutrophils were pre-treated or not with MEK/ERK1/2 kinase inhibitor (PD98059 (PD), 10 μM) for 1 h at 37°C followed by stimulation with LTB_4 (100 nM), its vehicle (ethanol; EtOH) or fMLP for 10 min After stimulation, β-hexosaminidase release was assessed.

n= 3

*=$P<0.05$

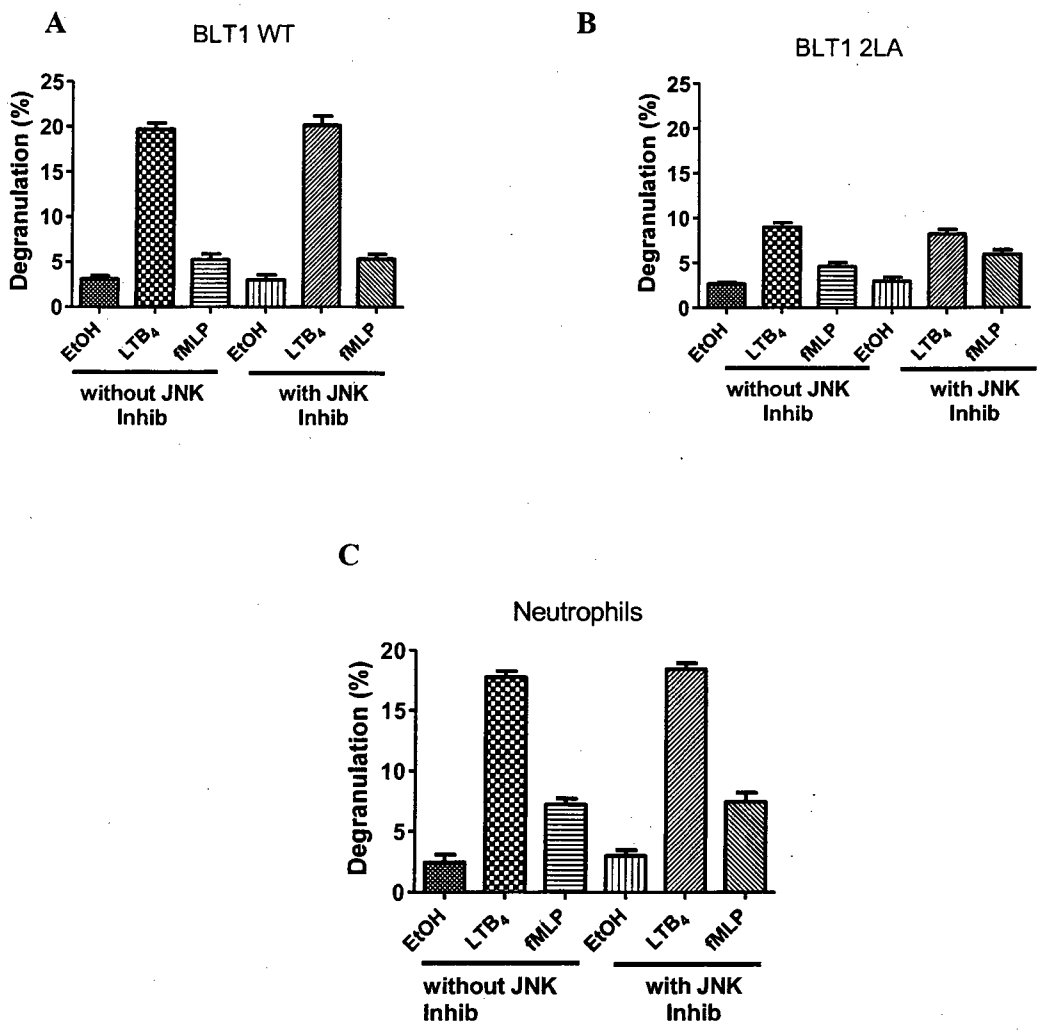


FIG 8: Effects of JNK kinase inhibition on LTB_4-mediated degranulation of PLB-BLT1 (wt), PLB-BLT1 (2LA) cells and human neutrophils

A) PLB-BLT (WT) cells (2.5 x 10^5 cells), B) PLB-BLT (2LA) cells (2.5 x 10^5 cells) and C) human neutrophils were pre-treated or not with a JNK inhibitor SP600125 for 1 h at 37°C followed by stimulation with LTB_4 (100 nM), its vehicle (ethanol; EtOH) or fMLP for 10 min After stimulation, β-hexosaminidase release was assessed.

n= 3

*=P<0.05

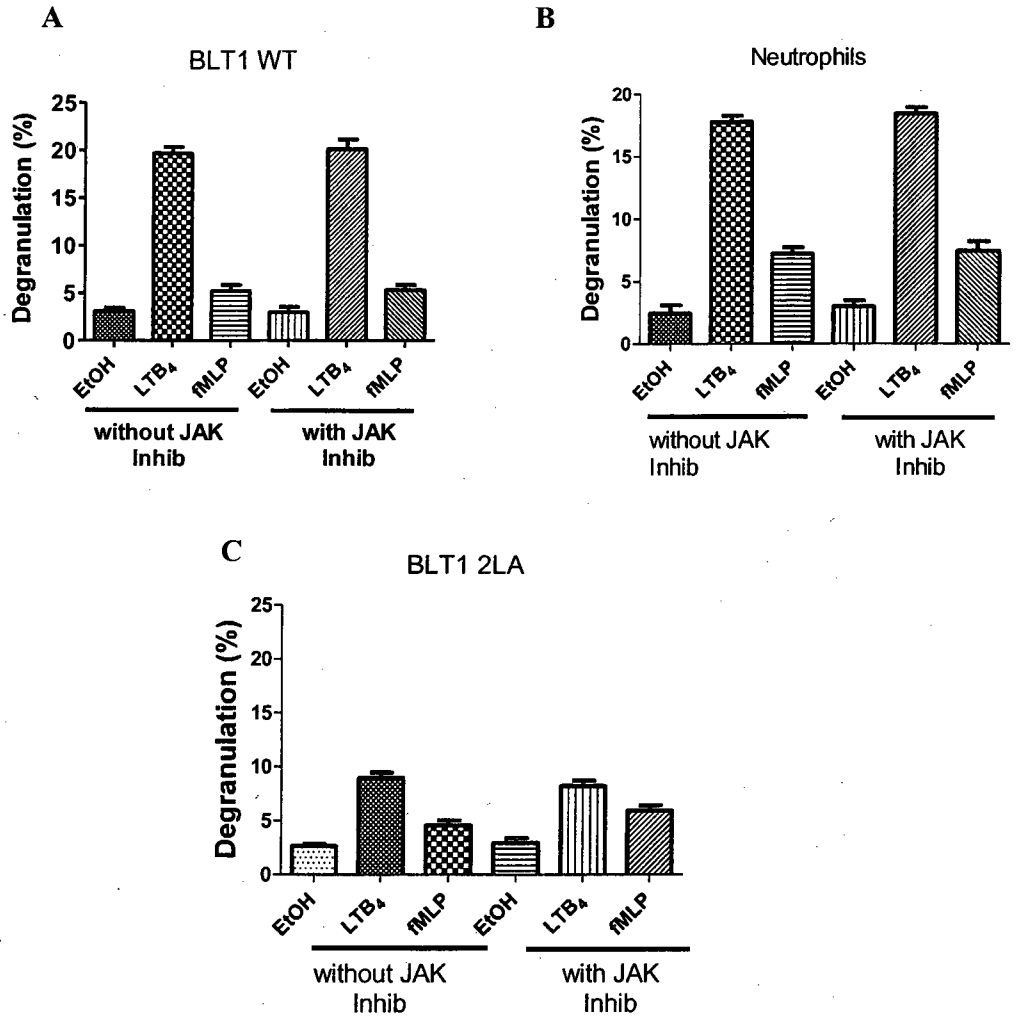


FIG 9: Effects of Jak kinase inhibition on LTB_4-mediated degranulation of PLB-BLT1 (wt), PLB-BLT1 (2LA) cells and human neutrophils

A) PLB-BLT (WT) cells (2.5×10^5 cells), B) PLB-BLT (2LA) cells (2.5×10^5 cells) and C) human neutrophils were pre-treated or not with a Jak kinase inhibitor (AG490 (AG), 10 μM) for 1 h at 37°C followed by stimulation with LTB_4 (100 nM), its vehicle (ethanol; EtOH) or fMLP for 10 min After stimulation, β-hexosaminidase release was assessed.

n= 3

*=$P<0.05$

RhoA activation is crucial for LTB4 mediated degranulation in neutrophils.

LTB4 signals through BLT1 and BLT2. Since several inhibitors known to inhibit degranulation through BLT1 failed to reduce the residual degranulation levels in the mutant BLT1-2LA cells. It was important to rule out the endogenous BLT2 expression and its influence on degranulation in mutant BLT1-2LA cells. PLB-BLT1 (wt) and PLB-BLT1 (2LA) cells were pre-treated with BLT1 antagonist U75302 (10 μM) and BLT2 antagonist LY2552833 (10 μM) for 30min and then stimulated with LTB4 for 10 min. We observed that degranulation was completely abrogated in both PLB-BLT1 (wt) (fig 10C) and PLB-BLT1 (2LA) (fig10D) with BLT1 antagonist, whereas BLT2 antagonist had no effect on BLT1 (wt) or on PLB-BLT1 (2LA) (fig10A-B).

RhoA and Rac1 activation by GPCRs has been shown to play an important role in neutrophil chemotaxis, since both granule mobilization and chemotaxis involve cytoskeleton re-arrangement. It was interesting to see the effect of the RhoA and Rac1 activation inhibition on LTB_4-mediated degranulation.

PLB-BLT1 (wt) and PLB-BLT1 (2LA) were pre-incubated with Rac1 inhibitor NSC23766 100μM for 1h (fig 11 A-B). No statistically significant reduction in degranulation was observed compared to PLB-BLT1 (wt) and PLB-BLT1 (2LA) stimulated with LTB_4 alone.

However when Human neutrophils, PLB-BLT1 (wt) and PLB-BLT1 (2LA) were pre-incubated with P160ROCK inhibitor Y-27632 (10μM) for 1h. A 50±5% reduction of β-hexosaminidase release in human neutrophils (fig12 C) and 70±5% reduction of β-hexosaminidase release in PLB-BLT1 (wt) cells was observed (fig9A). Whereas, Y-27632 inhibitor did not inhibit β-hexosaminidase release in PLB-BLT1 (2LA) mutant (fig 12 B). It was also observed that inhibition of β-hexosaminidase release by P160ROCK inhibitor, in PLB-BLT1 (wt) cells (fig 12 D) was concentration dependent, reaching its maximum inhibition at 10μM. Increase in the concentration of P160 ROCK inhibitor did not have an effect on PLB-BLT1(2LA) (fig 12 E).

To assess the extent of RhoA involvement in LTB_4-mediated degranulation, we performed a degranulation assay on PLB-BLT1 (wt) and PLB-BLT1 (2LA) cells that were transiently transfected with constitutively active RhoA or an empty vector (pcDNA3). Degranulation levels in PLB-BLT1(wt) cells (fig13 A) were similar to cells transfected with empty vector or those with constitutively active RhoA, whereas there was a 65±5% increase in degranulation levels of PLB-BLT1 (2LA) cells (fig13 B) transfected with constitutively active RhoA compared to PLB-BLT1(2LA) cells transfected with an empty vector.

Since the reduction in degranulation associated with the mutation of dileucine (304-305) motif in helix VIII can be corrected by transfecting cells with constitutively active RhoA, it appears plausible that the 2LA mutation caused the BLT1 to lose its capacity to activate

the Rho pathway. These results also provided a first indication that the Rho pathway may play a role in neutrophil degranulation.

Ongoing studies are concentrated on directly assessing whether BLT1 can activate RhoA and whether BLT1-2LA is deficient in this function.

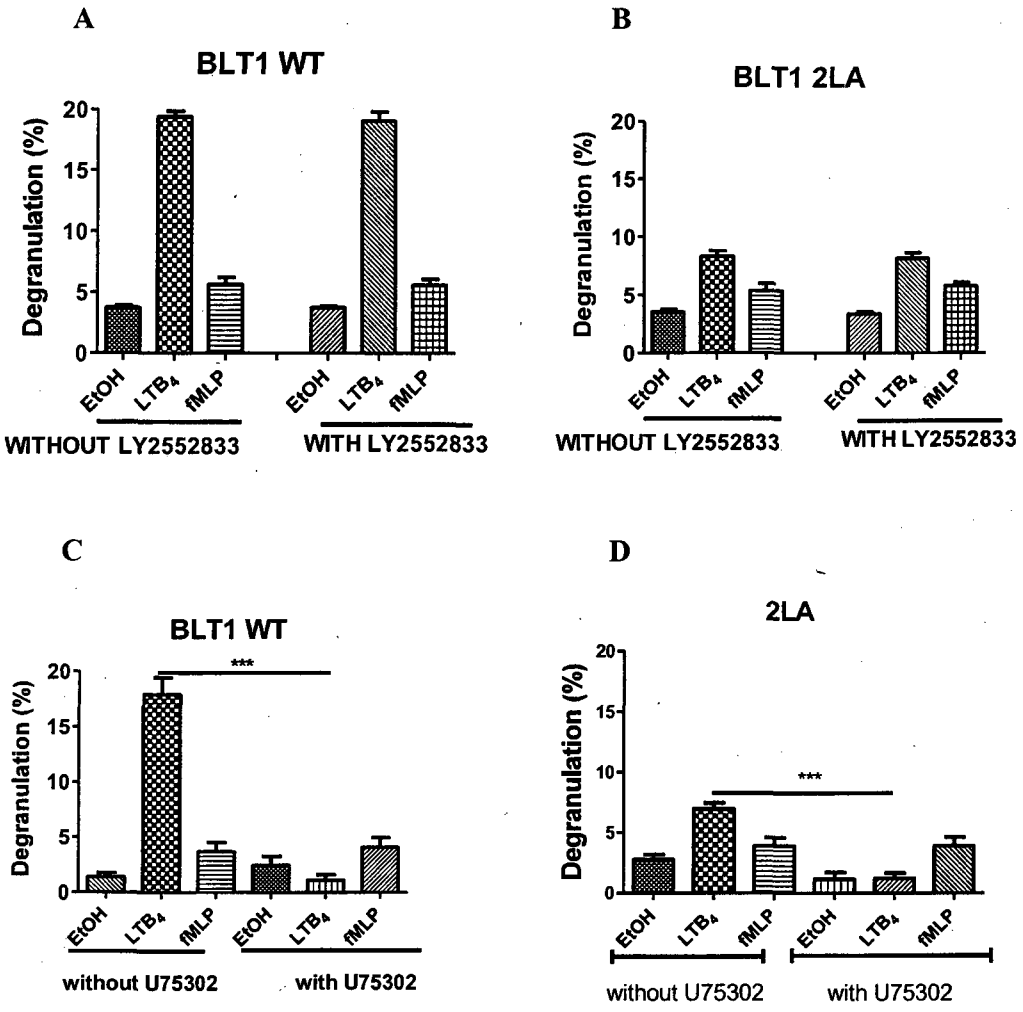


Fig 10: Effect of BLT1 and BLT2 antagonist on LTB4-induced degranulation.

PLB-BLT1 (WT and 2LA) cells (2.5 x 10^5 cells) were differentiated with DMSO from 0 to 3 days. Differentiated A) PLB-BLT (WT) cells and B) PLB-BLT(2LA) (2.5 x 10^5 cells) were pretreated or not with BLT2 antagonist LY2552833 (10 μM) at 37°C or C-D) BLT1 antagonist U75302 (10 μM) for 30 min before stimulation for 10 min with 100 nM LTB_4 or its appropriate vehicle (EtOH) or fMLP. After stimulation, β-hexosaminidase release was assessed.

n= 3

Fig C: ***=P<0.0001, fig D: ***=P<0.0010

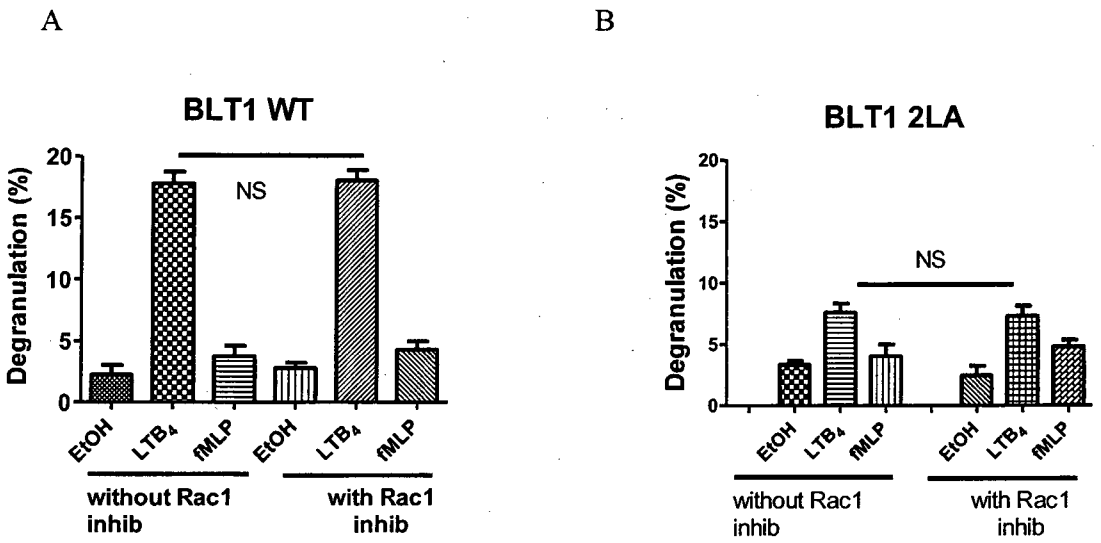
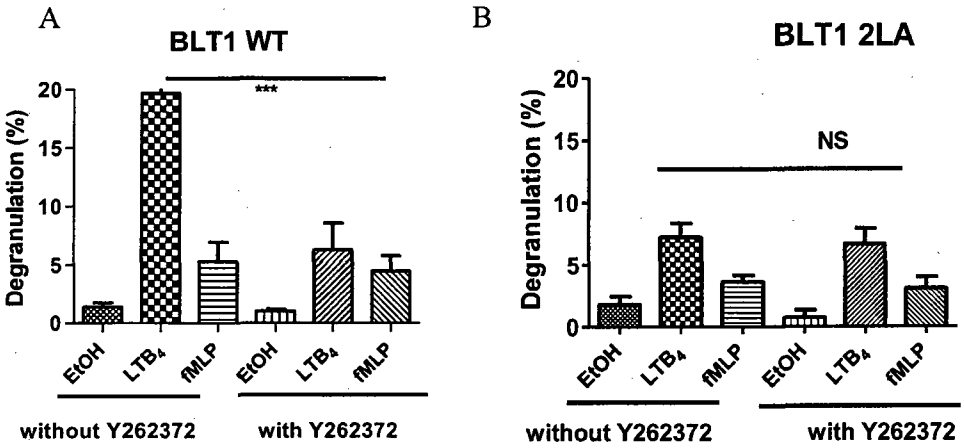
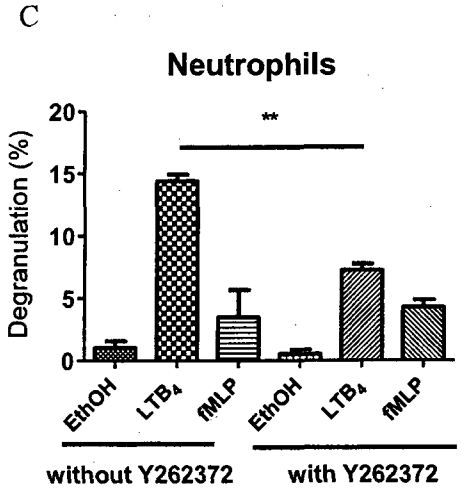


Fig 11: Effects of Rac1 inhibition on LTB_4-mediated degranulation in PLB-BLT1 (WT or 2LA)

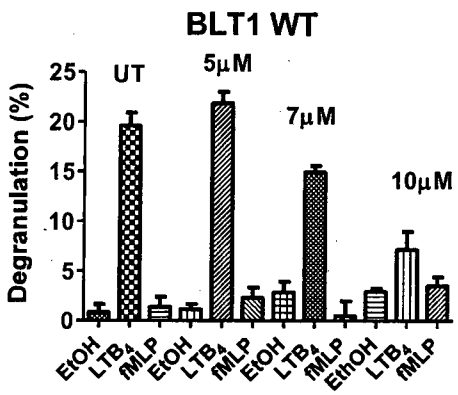
PLB-BLT1 (WT or 2LA) cells (2.5×10^5 cells) differentiated with DMSO from 0 to 3 days. Differentiated A) PLB-BLT (WT) cells and B) PLB-BLT(2LA) (2.5×10^5 cells) were pretreated or not with Rac1 inhib.NSC23766 100µM at 37°C for 1 hour before stimulation for 10 min with 100 nM LTB_4 or its appropriate vehicle (EtOH) or fMLP. After stimulation, β-hexosaminidase release was assessed.

n= 3


A
BLT1 WT
***
Degranulation (%)
20
15
10
5
0
EtOH
LTB4
fMLP
EtOH
LTB4
fMLP
without Y262372
with Y262372
B
BLT1 2LA
NS
Degranulation (%)
20
15
10
5
0
EtOH
LTB4
fMLP
EtOH
LTB4
fMLP
without Y262372
with Y262372


C
Neutrophils
**
Degranulation (%)
20
15
10
5
0
EthOH
LTB4
fMLP
EthOH
LTB4
fMLP
without Y262372
with Y262372

D



E

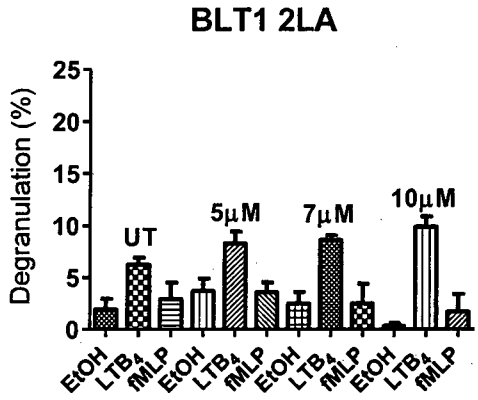


Fig 12: Effects of ROCK (P160 ROCK) inhibition on LTB_4-mediated degranulation in PLB-BLT1 (WT or 2LA) cells and human neutrophils.

PLB-BLT1 (WT or 2LA) cells (2.5×10^5 cells) were differentiated with DMSO from 0 to 3 days. Differentiated **A)** PLB-BLT (WT) cells and **B)** PLB-BLT (2LA) (2.5×10^5 cells) and (***C***) human neutrophils (3×10^6 cells) were pre-treated or not with Y62375 inhibitor (10 μM) at 37°C for 1 hour before stimulation for 10 min with 100 nM LTB_4 or its appropriate vehicle (EtOH) or fMLP. After stimulation, β-hexosaminidase release was assessed. **D)** Concentration-dependent inhibition of PLB-BLT1 (wt) degranulation using Y62375 inhibitor (0–10 μM). **E)** Concentration-dependent inhibition of PLB-BLT1 (2LA) degranulation using Y62375 inhibitor (0–10 μM).

n= ≥ 3

***=P<0.0004

**=P<0.0015

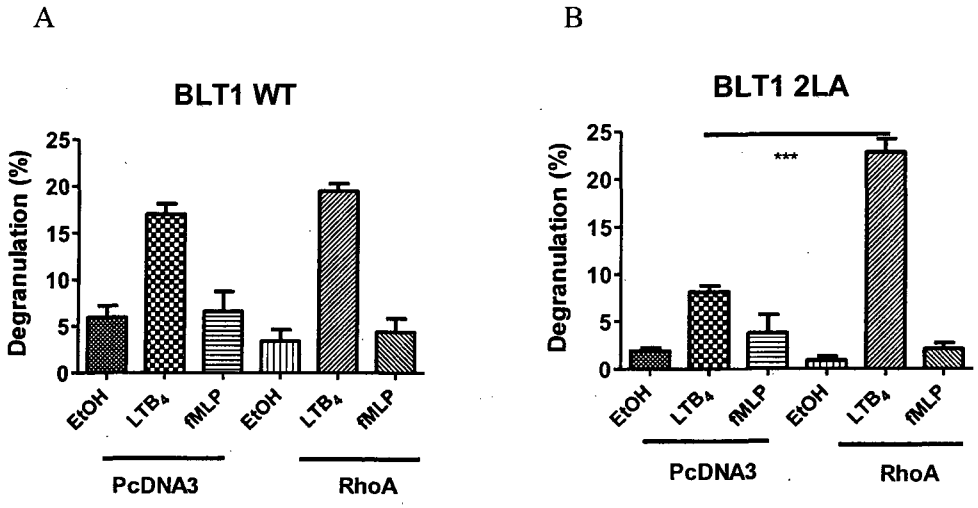


Fig13: Transfection of PLB-BLT1 (wt) and (2LA) mutant with constitutively active RhoA.

A) Differentiated PLB-BLT1 (WT) and B) PLB-BLT1 (2LA) cells were transfected with 10μM of constitutively active RhoA plasmid or an empty vector (pc DNA) and stimulated with 100 nM LTB_4 or its appropriate vehicle (EtOH) or fMLP. After stimulation, β-hexosaminidase release was assessed.

n= 3

***=P<0.0007

Discussion

In our study, we addressed the role of the helix VIII of BLT1 receptor in neutrophil degranulation and the intracellular signaling that follows the stimulation of BLT1 by LTB_4.

The following conclusions can be drawn from our study: (1) the neutrophil-like cellular model of PLB-BLT1 and PLB-BLT1 mutants is suitable for studying the effects of mutations on BLT1-mediated degranulation. (Smith, Borgeat et al.). (2) The distal dileucine motif in helix VIII of the BLT1 receptor plays an important role in LTB_4-mediated degranulation in neutrophils. (3) Pertussis toxin–sensitive Gαi/o protein subunit and PI3k and Src family kinases are involved in LTB_4-mediated neutrophil degranulation. (4) JAK2, p38 kinase and MEK/ERK kinases do not play a role in BLT1-mediated degranulation. (5) RhoA downstream signalization may be disrupted by the mutation of the distal dileucine motif leading to a considerable reduction in degranulation. The degranulation levels in mutant BLT1 cells were restored to the levels of human neutrophils and PLB-BLT1 (wt) degranulation, after the mutant PLB-BLT1 (2LA) cells were transfected with a constitutively active form of RhoA.

The promyeloid PLB-985 cell line has been shown to differentiate into neutrophil or monocyte-like cells (Tucker, Lilly et al. 1987). Pedruzzi et al. (Pedruzzi, Fay et al. 2002) used differentiated PLB-985 cells to study fMLP-mediated degranulation. PLB-985 cells were also used by Kaldi et al. (Kaldi, Kalocsai et al. 2003) to show that fMLP-mediated

degranulation was dependent on the presence of cholesterol in the cell membrane. Gaudreault et al. (Gaudreault, Thompson et al. 2005) studied the involvement of BLT1 endocytosis and Yes kinase activation in degranulation using PLB-985 cells. Similarly Lambert et al. used PLB985 cells to study the role of the distal dileucine motif of helix VIII and RhoA in LTB_4-mediated chemotaxis. Christophe Pivot-Pajot et al. (Pivot-Pajot, Chouinard et al. 2009) recently characterized degranulation and phagocytic capacity of PLB-985 cells.

Even though PLB-985 cell are shown to be a suitable model for neutrophils, an important step of our study was to compare the degranulation levels of stably transfected differentiated PLB-BLT1 (wt) cells with freshly acquired human neutrophils which can be later used as a standard of comparison with mutant PLB-BLT1 cells. Our results demonstrated that the degranulation levels of PLB-BLT1 (wt) cells were comparable to those of human neutrophils.

Okuno et al. (Okuno, Ago et al. 2003) demonstrated that BLT1 mutants with a truncated or substituted helix VIII showed much higher LTB_4 binding than wild-type (WT) receptor in HEK293 and CHO cells, despite having comparable expression on the cell surface. In addition Gaudreau *et al.* (Gaudreau, Le Gouill et al. 2002) proposed that Thr-308 is involved in GRK6-mediated desensitization of BLT1 signaling in which a C-tail-truncated BLT1 (G291stop) demonstrating an increased number of binding sites for LTB_4 as well as enhanced signal transduction in response to LTB_4 stimulation, whereas internalization was drastically reduced when compared with BLT1 (wt).

Gaudreau *et al.* (Gaudreau, Beaulieu et al. 2004) used mutational analysis of determinants Located in the C-terminal (C) tail of BLT1 and found that disruption of the distal dileucine (Leu^{304}-Leu^{305}) motif resulted in a mutant BLT1 with a higher number of binding sites on the cell surface, whereas the 2LA mutant BLT1 showed only a slight increase in the number of binding sites. Substitution of Leu^{304}-Leu^{305} appeared to prevent LTB_4-induced BLT1 internalization. Similarly Lambert et al. proposed that mutation of distal dileucine (Leu^{304}-Leu^{305}) of BLT1 disrupts the signalization of RhoA and leads to formation of multiple pseudopods, thus affecting chemotaxis whereas the addition of constitutively active RhoA corrected the defect.

Receptor internalization is an important part of downstream signaling, whereas cytoskeletal rearrangement plays a crucial role in granule movement in the process of degranulation. Our results demonstrated that substitution of dileucine motif with alanine in 2LA mutant lead to a reduction of 60-65% of degranulation levels compared to PLB-BLT1 (wt) and human neutrophils, suggesting that the distal dileucine motif plays an important role in BLT1 mediated neutrophil degranulation.

The reduction but not total abrogation of degranulation in PLB-BLT1 (2LA) mutant cells provided us with dual challenge 1) to determine the extent of the involvement of the dileucine motif in the process of degranulation and 2) to see if there is an alternative pathway involved which may be independent of the dileucine motif.

Signaling of BLT1 through $G\alpha i/_0$ protein subunit and its involvement in neutrophil degranulation has been shown before, Masuda et al. (Masuda, Itoh et al. 2003) using a reconstitution system made up of GPCR and heterotrimeric G proteins on extracellular baculovirus particles (budded virus (BV) showed that $G\alpha_i$ couples to BLT1. In addition Gaudreau et al. used Cos-7 cells to demonstrate that signaling through the leukotriene B_4 receptor involves $G\alpha_i$. Whereas Gaudreault et al. demonstrated that degranulation levels in differentiated PLB985 cells stably transfected with BLT1 were significantly reduced once treated with $G\alpha_{i/o}$ inhibitor PTX. Several studies have also demonstrated the relevance of PI3k in LTB_4 mediated degranulation.

Ito et al. (Ito, Yokomizo et al. 2002) using BLT1 rat basophilic leukemia cells (RBL-2H3) showed that PI3k was necessary for enzyme release. Similarly Gaudreau et al. demonstrated the importance of PI3k in degranulation using differentiated PLB-BLT1 cells.

All of these findings made $G\alpha_{i/o}$ and PI3k good candidates for testing the role of the distal dileucine motif (L304-L305) in the helix VIII of BLT1 receptor. The $G\alpha_{i/o}$ inhibitor PTX significantly reduced the degranulation levels in both PLB-BLT1 (wt) and human neutrophils, as had been shown before by Gaudreault et al, but it did not have any effect on residual degranulation levels of PLB-BLT1 (2LA) mutant with disrupted dileucine motif, indicating that intact distal dileucine motif (304-305) in helix VIII is essential for BLT1 to signal through $G\alpha_{i/o}$.

Our studies also demonstrated that PI3K is important for LTB_4 mediated degranulation in PLB-BLT1 (wt) and human neutrophils but the PI3K inhibitor did not have any effect on degranulation levels of PLB-BLT1 (2LA) mutant cells. Thus reiterating the fact that an intact dileucine motif in helix VIII is important for signaling through PI3K.

Src family kinases have already been connected to different cellular activities in human neutrophils, such as degranulation. Mocsai et al. (Mocsai, Ligeti et al. 1999) demonstrated that adhesion-dependent neutrophil lactoferrin release requires Fgr and Hck activity by using src kinase blockade with PP1 and neutrophils deficient in Fgr and Hck. Similarly Gaudreault et al. (Gaudreault, Thompson et al. 2005) has shown the involvement of src kinase in LTB_4-mediated degranulation using PLB-BLT1 cells.

As expected our results also indicated significant reduction in degranulation levels of PLB-BLT1 (wt) cells but Src inhibitor did not have any effect on degranulation levels of PLB-BLT1 (2LA) mutant cells. Our results confirmed the involvement of Src family kinase

Since several inhibitors known to inhibit degranulation failed to reduce the residual degranulation levels in PLB-BLT1 (2LA) mutants it was important to rule out the involvement of BLT2 receptor. Using BLT1 and BLT2 antagonists we confirmed that the residual degranulation was indeed due to signaling through BLT1receptor.

The BLT2 antagonist had no effect on PLB-BLT1 (wt) or PLB-BLT1 (2LA) mutant cells degranulation levels whereas BLT1 antagonist completely abrogated degranulation in both the wt and 2LA mutant cells.

Receptor endocytosis is an important step for BLT1 signaling and degranulation (Gaudreault, Thompson et al. 2005), knowledge of the fact that disruption of the dileucine motif significantly reduces receptor internalization (Gaudreau, Beaulieu et al. 2004) suggested that there may be an alternate path way involved which is independent of internalization and does not signal through conventional pathway.

The prospect of an alternate pathway led us to venture in direction not previously explored in degranulation. MAPK family is known to regulate several important neutrophil functions. In addition Downey et al (Downey, Butler et al. 1998) proposed that exposure of neutrophils to inflammatory stimuli such as the chemoattractant fMLP leads to activation of MEK. Wozniok et al.(Wozniok, Hornbach et al. 2008) showed that Inhibition of the ERK signaling pathway abolishes neutrophil migration induced by C. albicans filaments selectively impairing its ability to kill. However our results suggest that MEK/ERK inhibitor did not produce a significant reduction in degranulation levels of PLB-BLT1 (wt) or PLB-BLT1 (2LA) mutant cells removing it from our list of suspects responsible for PLB-BLT1 (2LA) mutant's residual degranulation. Similarly our results excluded JNK and P38 the other two members of MAPK family from the list of perpetrators.

Several GPCRs are capable of signaling without internalization. Frank et al. (Frank, Saito et al. 2002) proposed that Angiotensin II receptor can activate JAK2. In addition Lukashova et al. (Lukashova, Chen et al. 2003) demonstrated that PAFR is capable of signaling through JAK2 without internalization and Gaudreau *et al.* (Gaudreau, Beaulieu et al. 2004) has shown that receptor internalization in PLB-BLT1 (2LA) mutant cells was significantly reduced. To see if PLB-BLT1 (2LA) mutant cells were signaling in similar fashion as Angiotensin II receptor or PAFR and if the JAK/STAT was at play here, we used JAK2 inhibitor AG490. Our result excluded the involvement of JAK2.

The major function of Rho GTPases is to regulate the assembly and organization of the actin cytoskeleton (Hall 1998). Some common target proteins appear to be utilized by both Rac and Cdc42 in the induction of lamellipodia and filopodia respectively (Manser, Leung et al. 1994; Manser, Chong et al. 1995; Bagrodia, Taylor et al. 1995), however our results indicated that both Rac1 and cdc42 (results not shown) were not involved in the signaling of residual degranulation of PLB-BLT1 (2LA) mutant cells.

ROK p160 (also known as Rho-associated coiled-coil-containing protein kinase or ROCK) appears to be required for Rho-induced assembly of stress fibres and focal adhesions (Ishizaki, Maekawa et al. 1996; Ishizaki, Naito et al. 1997). Expression of the constitutively active catalytic domain of ROKa induced stellate actin-myosin filaments in HeLa and Swiss3T3 cells (Leung, Chen et al. 1996; Ishizaki, Naito et al. 1997).

Inhibition of ROK, on the other hand, using a pharmacological inhibitor (Y-27632) caused loss of serum and [Val^{14}] Rho-induced stress fibres (Uehata, Ishizaki et al. 1997). Lambert et al. has shown that pretreatment of differentiated PLB-BLT1 cells with p160 Rock (Y-27632) formed multiple pseudopod indicating defective chemotaxis. In our study we pretreated or not differentiated PLB-BLT1 (wt) and PLB-BLT1 (2LA) mutant cells with p160 Rock (Y-27632) inhibitor. Our results indicated involvement of the RhoA in degranulation process as pretreatment with the inhibitor caused 70% reduction degranulation levels PLB-BLT1 (wt) cells but the inhibitor did effect the residual degranulation levels of PLB-BLT1 (2LA) mutant cells.

To further investigate the effect of the defect in the dileucine motif, we transfected differentiated PLB-BLT1 (wt) and PLB-BLT1 (2LA) cells with constitutively active RhoA. Interestingly enough the degranulation levels in PLB-BLT1 (2LA) mutant cells were restored to PLB-BLT1 (wt) levels. The transfection had no effect on PLB-BLT1 (wt) degranulation levels suggesting that RhoA activation is crucial for degranulation process and the dileucine motif in helix VIII is essential for activation of RhoA and downstream signaling.

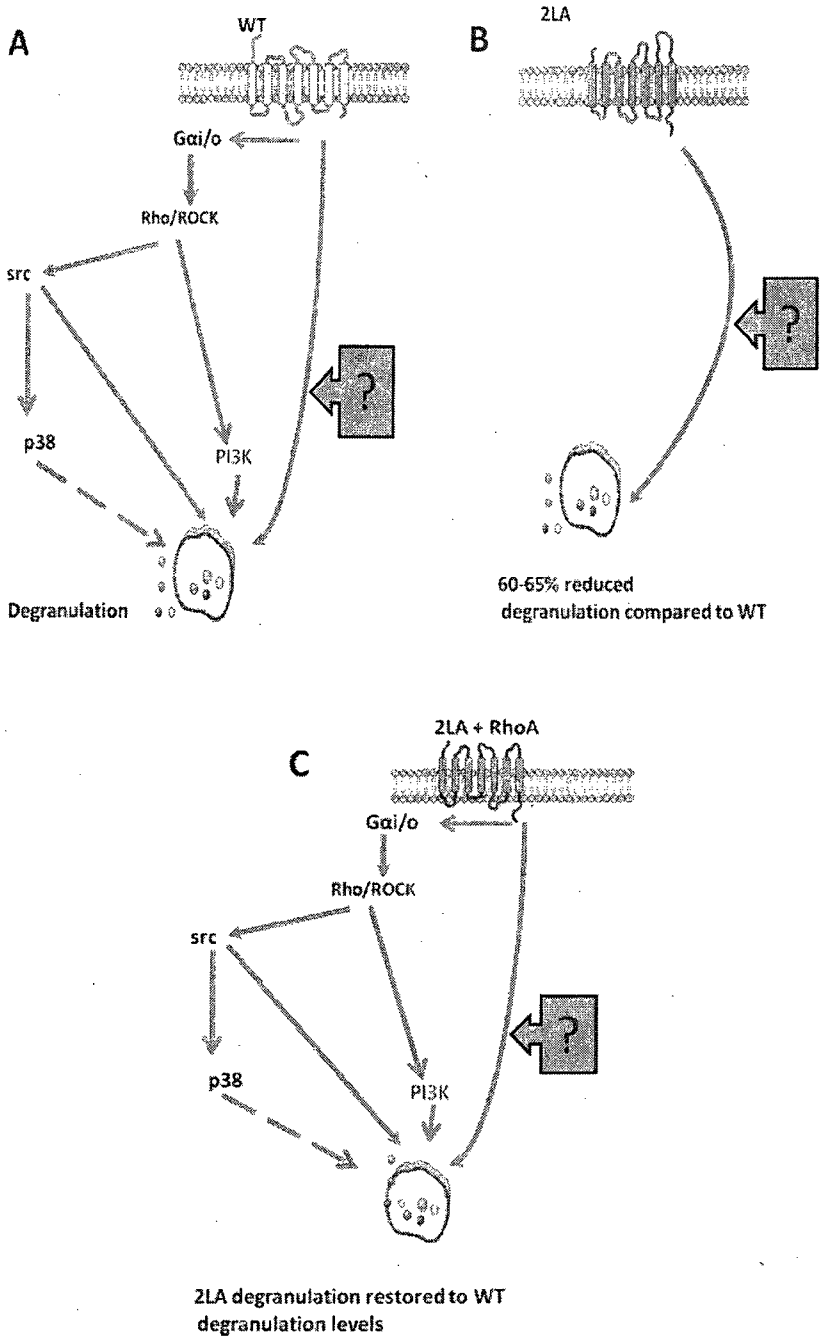


Diagram 1: suspected pathway of degranulation in A: WT, B: 2LA and C: 2LA transiently transfected with constitutively active RohA.

Our study has implicated src family kinases, PI3K and RhoA in LTB_4-mediated degranulation; the sequence in which these components are activated is still unclear. Further studies are also required to confirm binding of RhoA to the dileucine motif in helix VIII, since it remains to be seen if RhoA/ROCK is activated by helix VIII or by an alternative mechanism.

Insight into mechanisms regulating degranulation of neutrophils could enable us to specifically modify granule recruitment and exocytosis of neutrophils at the site of infection. This could lead to novel therapeutic strategies for counteracting neutrophil-induced tissue damage, for example, in sepsis, asthma, ischemia/reperfusion injury and transplant rejection

Conclusion

Neutrophil degranulation is an important part of immune system. Lack of or defect in neutrophil degranulation can have serious consequences. Our results suggest that RhoA activation is crucial for neutrophil degranulation and the distal dileucine motif (304-305) plays an important role in RhoA activation. In addition the residual degranulation suggests that there may be other pathways involved in LTB_4-mediated degranulation which are independent of RhoA activation and receptor internalization.

Similarly our results indicate that $G\alpha_i/_o$ Protein subunit, PI3K and src family kinases play a role in degranulation signaling. However the results suggest that they are activated downstream of RhoA. In addition our results also demonstrate that JAK2, p38, MEK/ERK and JNK do not play a role in LTB_4-mediated degranulation.

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